Chapter 14

Innovative Approaches to Develop Prophylactic and Therapeutic Vaccines against HIV/AIDS

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Abstract

The acquired immunodeficiency syndrome (AIDS) emerged in the human population in the summer of 1981. According to the latest United Nations estimates, worldwide over 33 million people are infected with human immunodeficiency virus (HIV) and the prevalence rates continue to rise globally. To control the alarming spread of HIV, an urgent need exists for developing a safe and effective vaccine that prevents individuals from becoming infected or progressing to disease. To be effective, an HIV/AIDS vaccine should induce broad and long-lasting humoral and cellular immune responses, at both mucosal and systemic level. However, the nature of protective immune responses remains largely elusive and this represents one of the major roadblocks preventing the development of an effective vaccine. Here we summarize our present understanding of the factors responsible for resistance to infection or control of progression to disease in human and monkey that may be relevant to vaccine development and briefly review recent approaches which are currently being tested in clinical trials. Finally, the rationale and the current status of novel strategies based on nonstructural HIV-1 proteins, such as Tat, Nef and Rev, used alone or in combination with modified structural HIV-1 Env proteins are discussed.

Introduction

Epidemiology: Main Global and Regional Trends

Human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) remains one of the most serious threats not only to global health, but also to global development. According to the 2007 AIDS epidemic update by World Health Organization (WHO) and the Joint United Nations Programme on HIV/AIDS (UNAIDS), approximately 33.2 million people were living with HIV in 2007[1] of whom 2.5 million were due to new infection among adults and children (Fig. 1). Today, Sub-Saharan Africa continues to pay the highest toll to the global epidemic, with 68% of new infections, 76% of the estimated 2.1 million deaths in 2007 and 90% of the 2.5 million children living with HIV worldwide. Despite major methodological improvements, which have cut by 16% the former estimates, the statistics still indicate that the number of people living with AIDS increased in 2007, partly due to expanded access to therapy that reduced the number of deaths. This new analysis also shows that the pandemic actually peaked in the late 1990s and

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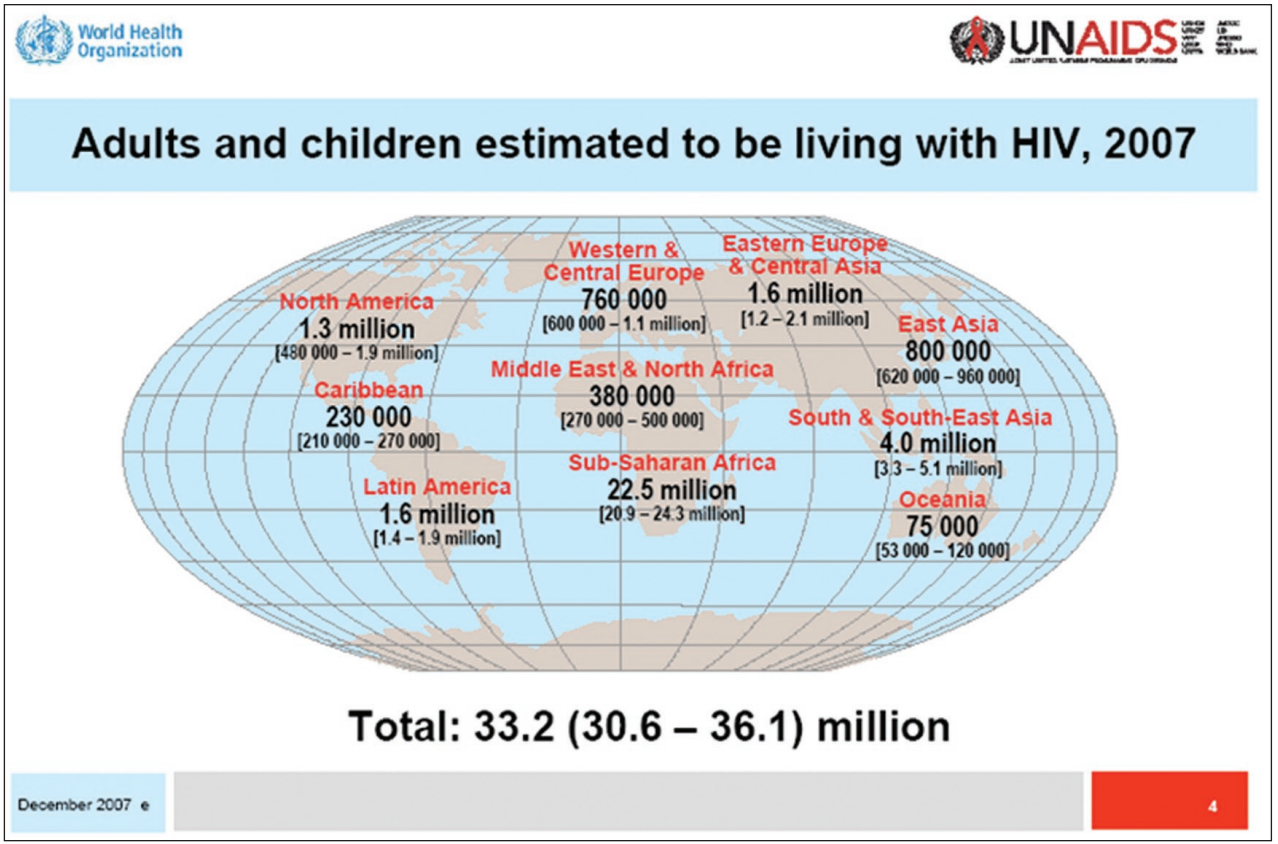

Figure 1. Adults and children estimated to be living with HIV, 2007. Reproduced by kind permission of UNAIDS (2007) (www.unaids.org).

the subsequent decline may at least in part be attributed to the efficacy of prevention campaigns. While this is certainly good news, it has important practical consequences on clinical trials, which may actually result undersized and lack statistical power. As today, longitudinal prospective studies appear the only way to realistically measure the incidence and will have to be included in the preparatory studies for vaccine testing.[2]

Rationale and Roadblocks to HIV Vaccine Development

The increasing social and economical, including therapy, costs of the pandemic make the search for a preventive and therapeutic HIV/AIDS vaccine the highest priority of the world HIV/AIDS agenda.[3] Preventive vaccination is aimed at inducing protective immune responses in individuals naive to HIV, whereas therapeutic vaccination is aimed at increasing the potency and breadth of anti-HIV immune responses in order to avoid or delay anti-retroviral therapy use in HIV-1 infected individuals.[4]

Despite advancements in the understanding of HIV biology and pathogenesis[5] and despite over 20 years of attempts, no vaccine is available to combat the HIV/AIDS epidemic.[6,7] However, the vast majority of vaccines evaluated so far turned out to be safe and immunogenic, with some of them showing some level of protection in preclinical models and therefore advanced to clinical testing.[8,9]

The difficulty in generating an HIV vaccine capable of eradicating the infection depends on many factors. Among the most important, the high genetic variability of HIV (for a review see refs. 10,11), the peculiar properties of the envelope (Env) proteins to avoid and evade immune recognition and neutralization,[12] the lack of identified correlates of immune protection, the complexity of implementing preclinical animal models[1,13,14] and conducting efficacy clinical trials, especially in developing countries. In addition to the elusion of antibody (Ab) neutralization, HIV is able to elude cellular immune responses by multiple mechanisms, including down-regulation of major histocompatibility complex (MHC) class I molecules,[15] emergence of mutants escaping cytotoxic T-lymphocytes (CTLs)[16] and dysregulation of cytokine production.[17-19]

Correlates of Protection

Lessons from the Natural History of HIV-1 and SIV Infection

The comprehension of mechanisms of natural resistance to HIV/AIDS may have implications for the identification of novel anti-viral strategies and in particular for the development of innovative diagnostics, therapeutics and vaccines. It is now clear that both host and viral factors contribute to the outcome of the infection and may explain the higher or lower individual susceptibility to HIV/AIDS (Table 1). In particular, two phenomena of natural resistance to HIV-1 infection or progression to disease have been described: individuals that remain uninfected despite exposure to the virus [multiply exposed uninfected (MEU) individuals, also termed exposed-uninfected (EU), or highly exposed persistently seronegative (HEPS) individuals][20] and individuals that become infected but do not progress to AIDS [long-term nonprogressors (LTNPs)]. Both MEU and LTNPs offer valuable clues to elucidate immune mechanisms involved in the resistance or control of infection, respectively and might thus provide a unique resource to identify correlates of protective immunity to HIV.

MEU include HIV-discordant couples having unprotected sex,[21,22] sex workers[23-25] and health care workers.[26,27] Homozygosis for a mutation in CCR5 gene (the 32 bp deletion, i.e., CCR5-Delta32 allele) is presently considered the most relevant genetic protective factor[28-30] (Table 1). Further, in a cohort of MEU Kenyan sex workers HIV-1 specific CTLs were found both in the blood and in the vaginal mucosa.[31] Interestingly, these CTLs, which had similar specificities, recognized epitopes distinct from those recognised by HIV infected individuals[24] and may contribute to protection from infection with both cytolytic and noncytotoxic anti-viral effector mechanisms.[32,33] In the Nairobi cohort, HIV-1 specific $CD4^+$ T-cells produce interleukin-2 (IL-2) rather than interferon-γ (IFN-γ) and display limited activation and cell death as compared to HIV-1 infected

Table 1. Major host factors associated to resistance or control of HIV infection and relevant to vaccine development

Level		Resistance	Control	Reference
Genetic Polymorphism				
-	CCR5	CCR5 Δ32 homozygosis	CCR5 Δ32 heterozygosis	28,60,61
-	CCR2		CCR2-64I	473
-	CCR5 & CCL3L1		Polymorphisms of these genes affect the capacity to mount immune responses (DTH)	79
-	MHC	A2/6802 supertype	B57, B5801, B27 and additional polymorphisms in B, C and outside B and C loci	62,80,412
-	KIR		Activating (KIR3DS1) and inhibiting (KIR3DL1) NK cell receptors binding HLA-B molecules with isoleucine at position 80 (HLA-Bw4)	66,474
Innate Immune Response				
-	Natural antibodies	Against CCR5	Against CCR5	475,476
-	α and β defensins	Increased in the genital mucosae		36
-	CCL5 (RANTES)	Increased in the genital mucosae		477
-	NK	Increased production of IFN-γ	Conserved lytic activity despite altered phenotype	35,478
Adaptative Immune Response				
-	Antibodies	Neutralizing IgA in the genital mucosae		37,479
-	$CD4^+$ T-cells	Prevalent IL-2 production, limited activation and cell death as compared to infected individuals	Polyfunctional, high per cell cytokine production	480
-	$CD8^+$ T-cells	Polyfunctional, found also in the genital mucosae, with specificities different from those found in infected individuals	Polyfunctional, mostly against Gag, highly cytotoxic	34,63,71

CCR5: chemokine (C-C motif) receptor 5; CCR5 Δ32: a 32-base pair deletion mutant of the CCR5 gene; CCR2: chemokine (C-C motif) receptor 2; CCL3L1: C-C chemokine ligand 3-like 1 (MIP-1α isoform), a high affinity ligand for CCR5; MHC: Major Histocompatibility Complex; KIR: Killer cell Immunoglobulin Receptor; CCL5: Chemokine (C-C motif) ligand 5; NK: Natural Killer cells.

individuals[34] (Table 1). In addition, an increased production of IFN-γ by natural killer (NK) cells was found in these sex workers,[35] which, together with increased levels of chemokines and alpha and beta-defensins (for a review see ref. 36), indicate a substantial role for innate immunity in the observed protection. Abs may also have an important role since in MEU HIV-1-specific IgA but not IgG were detected in the genital mucosae, which displayed neutralizing activity[37] (Table 1).

Taken together, these data suggest that local and to a lesser extent systemic, innate and adaptive immune responses may develop upon sexual intercourses with HIV-1 infected partners, which protect from becoming overtly infected. Intriguingly, quitting sex work resulted in diminished CTL frequency and increased risk of infection, suggesting that long-term memory was not induced and persistent antigenic stimulation was required to maintain protective CTL responses[24] or IgA.[37,38] This short-term immunity may also be consistent with allogeneic responses triggered by sexual intercourses with multiple partners, which have been shown to confer transient immunity,[39,40] an observation that has led to the proposal of alloimmunization for HIV-1 vaccine design.[41]

LTNPs are HIV-1 infected people that remain clinically healthy for over 10 years with low (50-2,000 RNA copies/ml plasma, also defined as "controllers") to undetectable (<50 RNA copies/ml plasma, also defined as "elite controllers") viral load and a minimal loss of $CD4^+$ T-cells in the absence of any anti-retroviral therapy.[42-45] Nonprogression appears to depend on multiple factors. Some LTNPs have mutations in viral genes that influence replication and/or immune control, such as those coding for the regulatory and accessory proteins Nef, Rev, Tat, Vif, Vpr and Vpu.[46-52] The LTNPs harboring viruses with mutations in *nef* are of special interest because of the reported efficacy of vaccination of macaques with an attenuated simian immunodeficiency virus (SIV) carrying a deletion in the *nef* gene (SIVΔnef).[53-55] However, follow-up of the well-characterized Sidney Blood Bank Cohort revealed progression in 3 out of the 8 individuals infected with the very same HIV.[56] Of note, progression was observed also in newborns and a few adult macaques infected with the SIVΔnef.[57] Thus, HIV-1 (and SIV) carrying a mutation in *nef* (and long-terminal repeat) are not safe and, more importantly, the immune responses induced by these attenuated viruses are not effective at efficiently controlling the infection over time. Further attenuations of the SIVΔnef to ameliorate their safety profile reduced the degree of protection from superinfection,[58] indicating that much work needs to be done to implement this kind of vaccine.[59]

As many as 25-30% LTNPs may have specific genetic polymorphisms in the HIV-1 coreceptors CCR5 and CCR2, as well as in the gene encoding the CCR5 ligand macrophage inflammatory protein 1-α, which are associated with an impairment of viral attachment and thus infectivity[60,61] (Table 1).

Certain MHC haplotypes (B57, B5801 and to a lesser extent B27) are over-represented in LTNPs[62] and, together with a lower total magnitude and breadth of HIV-specific CTL responses preferentially targeting Gag, significantly correlate with nonprogression.[63] Of interest, human leukocyte antigen (HLA)-B restricted CTLs to HIV are of low avidity, express low levels of the exhaustion marker PD-1 and accounts for the vast majority of polyfunctional CTLs, confirming the relevance of polyfunctionality (especially IFN-γ and IL-2, see below) and shedding some light on the protective role played by HLA-B alleles.[64] The highest frequency of these low avidity, polyfunctional CTLs was observed in $B57^+$ individuals, which is over-represented in LTNPs. This is somewhat paradoxical in view of the known capability of HIV to downregulate through Nef the expression of MHC-I A and B molecules hampering therefore target recognition especially by low avidity CTLs.[65] However, since B57 is also a ligand for the inhibitory NK receptor termed KIR3DL1, which has recently been shown to strongly associate with nonprogression,[66] it has been proposed that the HIV-driven MHC downregulation may relieve this inhibition, triggering NK cell activity against the virus.[67] It is conceivable that, under these opposing pressures, HIV be forced to limit the degree of MHC downregulation to avoid NK surveillance, thus explaining the persistence of low avidity polyfunctional CTLs, which however keep in check the virus. It will be of interest to determine whether these CTLs are over-represented in LTNPs infected with viruses carrying mutations in Nef that hamper MHC downregulation. Taken together, these findings and

hypotheses suggest that NK cells actively participate with CTLs to the containment of infection and ways to stimulate this arm of the innate immunity should be considered in vaccine design.

Concerning the enhanced and qualitatively different polyfunctional activity [simultaneous production of two or more cytokines (TNF-α, IL-2, IFN-γ, MIP1-β) in conjunction with markers of degranulation, such as CD107a expression] of HIV-specific $CD8^+$ T-cells from LTNPs,[68] the capability of these T-cells to coproduce IL-2 appears the most important feature, possibly related to its ability to sustain proliferation, expanding the number of effector cells.[69,70] Along the same line, virus control has been associated with high frequencies of HIV-specific CTLs with a "nonexhausted" phenotype ($HLA\text{-}DR^{High}$, $CD38^{Low}$), which is consistent with retention of polyfunctionality and a low degree of immunoactivation and a potent ex vivo cytotoxic activity against autologous infected $CD4^+$ T-cells.[71] The importance of polyfunctionality is also suggested by in vitro experiments showing that fully competent autologous monocyte-derived dendritic cells (MDDCs) pulsed with Gag were capable to trigger the expansion of $CD8^+$ T-cells in LTNPs but non in progressors, whose $CD8^+$ T-cells were unable to produce IL-2.[72] These data should be carefully considered when designing therapeutic vaccine trials. In addition to the cytolytic activity, HIV-specific $CD8^+$ T-cells have been reported to secrete unknown anti-viral soluble factor(s) (for a review see ref. 73) that has been associated with nonprogression.[74,75]

$CD4^+$ T-cells are pivotal for induction and maintenance of effective T- and B-cell responses (for a review see ref. 76). Of interest, $CD4^+$ T-cells from individuals that control infection are polyfunctional and secrete higher amount of cytokines (IFN-γ, TNF-α and IL-2) as compared to monofunctional cells.[77] These findings highlight the importance of multi-parametric analysis of HIV-specific immune responses in order to correctly identify the functional properties relevant to protection or control of infection. However, whether these immunological features play a key role in controlling the infection or merely reflect a preserved immune system remains to be elucidated. To gain more insights into host genetic factors impacting control of infection, Consortia are being formed to allow analyses of large cohorts of patients.[78-80] Results from these studies indicate that control of infection is strongly influenced by host genetic factors, of which only some are related to adaptive immunity, underscoring the present need to search and identify novel immunological mechanisms (Table 1).

Despite a broader neutralizing antibodies (NAbs) response was reported in LTNPs as compared to patients with progressive disease,[81,82] no apparent role for NAbs in suppression of HIV replication could be demonstrated in elite controllers as well as in patients responding to HAART.[83] Nevertheless, plasma from LTNPs has been recently screened with random peptide phage libraries in order to identify mimotopes capable of inducing relevant NAbs against conformational epitopes.[84]

Another valuable model to investigate correlates of protection is represented by SIV infection in the natural host in which the virus typically does not induce AIDS despite chronic high levels of virus replication. A better understanding of the mechanisms underlying the lack of disease progression in African green monkeys (AGM)[85] and sooty mangabey monkeys (SM) may provide clues to the pathogenesis of immunodeficiency in HIV-infected humans.[86-88] Recent findings support the idea that in the natural host a strong and rapid immunosuppressive response mediated by regulatory T-cells (Tregs) abrogates immune hyperactivation, resulting in a more benign disease outcome.[89,90] Since control of infection appears to occur early after infection, natural immunity is also believed to be key, either by combating directly the virus, or by instructing an effective adaptive immune response.[91] Strikingly, protection from progression may occur in these animals despite severe loss of $CD4^+$ T-cells both in the blood and in the mucosae, indicating that $CD4^+$ T-cell depletion per se is not sufficient to drive progression and that these species have evolved mechanisms to compensate for the T-helper loss.[92-94] Low levels of T-cell activation and apoptosis, preserved lymph nodes architecture and no sign of immunodeficiency were the hallmarks of nonprogression in these monkeys. Taken together, the findings in human and nonhuman primates suggest that blunting of initial inflammatory response and immune hyperactivation correlate with maintenance of polyfunctional T-cells and lack of phenotypes associated to progression (loss of

polyfunctionality and especially of IL-2 production, upregulation of CD38, PD-1, CTLA-4, downregulation of CD107) and should therefore be the goals of vaccines aimed at containing the virus. As a corollary, immunization strategies resulting in sustained vaccine antigen load and immune hyperactivation may be detrimental. Finally, some concerns about the criteria used to define protection as control of infection. In fact, the above studies in the natural hosts as well as those in vaccinated animals[95,96] question the concept that plasma viremia levels and $CD4^+$ T-cell counts are always reliable indicators of vaccine efficacy. Extended follow-up of "bona fide" protected animals is therefore recommended.

What We Have Learned from Vaccination Studies

Although the correlates of protective immunity are still elusive,[97,98] there is substantial evidence that preventive and therapeutic vaccine approaches elicit both humoral (broadly NAbs)[99] and cell-mediated (T-helper and -cytotoxic) responses[100] (Table 2). NAbs are believed not to play a pivotal role in early HIV-1 clearance in the natural infection, since they appear when substantial destruction of the gut associated lymphoid tissue (GALT) has already occurred and the virus has colonized virtually every tissue.[101,102] Conversely, it is conceivable that the induction of a robust Ab response by vaccinating prior to exposure to the virus may protect from infection.

In order to prevent infection by a diverse range of HIV-1 isolates, a vaccine must elicit Abs that are broadly neutralizing. The HIV Env glycoproteins gp120 and gp41 mediate binding and entry into target cells and are the main viral targets for NAbs. However, the domains of gp120 and gp41 which are critical to cell attachment and entry are hidden from the attacking Abs, thus enabling HIV to evade neutralization.[103]

Nevertheless, a few monoclonal Abs (mAbs) with broad neutralizing activity have been identified in HIV-1 positive patient sera and well characterized (2F5, 4E10, 2G12 and b12).[104-106] Passive transfer of these mAbs protected neonatal macaques from subsequent challenge with SIV[107] and delay viral rebound after anti-viral treatment interruption in acutely and chronically infected patients.[108] However, polyspecific reactivity with autoantigens, reported for 2F5 and 4E10, the two NAbs targeting the membrane proximal external region of gp41, suggests that their production might be regulated by tolerance mechanisms.[109] This raises doubts about the feasibility and safety of inducing in healthy individuals Abs with these specificities.[110]

Although NAbs do not appear to contribute to the control of viremia in acute HIV-1 infection, Abs to the Env can be detected at the time of reduction of plasma viremia and other effector functions of Abs may play a role in viral clearance.[111] In fact, significant reduction of set-point viral loads and preservation of central memory $CD4^+$ T-lymphocyte counts were observed in rhesus macaques challenged with a pathogenic SIV (SIV_{mac239}) and passively immunized 7 days later with SIV_{mac239} specific NAbs, despite the rapid disappearance of the NAbs from the plasma.[112] Further, recent data indicate that HIV-1 infected primary cells and in particular macrophages and dendritic cells (DCs), are more susceptible than cell lines to Ab effector functions, including neutralization,[113] suggesting that the effectiveness of the Ab responses elicited so far by vaccination might have been underestimated. Moreover, there is evidence that immunization may induce memory B-cell responses that can last for several years and that can be boosted upon recall vaccination.[114,115]

Finally, antibody-dependent cell-mediated cytotoxicity (ADCC) has recently been reconsidered as an important Ab effector function that may contribute to HIV control (for a review see ref. 116). ADCC is an immune mechanism in which Abs bind target cells, making them vulnerable to the attack by immune cells carrying receptors for the Fc portion of the Ab (FcR) such as NK cells, γδ T-cells, neutrophils and macrophages. Although ADCC against HIV and SIV has been reported since many years,[117-123] only recently it was shown to correlate with protection against SIV in a prime/boost AIDS vaccine approach in rhesus macaques.[124] More recently, the protection afforded by passive immunization with the broadly neutralizing mAb b12 against an intravaginal challenge with the R5-tropic $SHIV_{SF162P3}$ was strongly diminished in monkeys immunized with a b12 variant devoid of FeR binding activity,[125] underscoring the relevance of additional Ab effector functions even in the context of neutralization. This is of importance for several reasons: (i) Abs

Table 2. Key scientific issues to successfully develop an Env-based vaccine according to a Working Group convened by the Global HIV Vaccine Enterprise[†]

Structure-Assisted Immunogen Design	- To improve understanding of the structural basis of antibody binding to the HIV-1 Env glycoprotein
	- To stabilize gp120 into more immunogenic forms or to scaffold conserved neutralization epitopes into foreign proteins
	- To identify epitopes capable of inducing neutralizing antibodies or antibodies contributing to neutralization (additive or synergistic effect)
To Assess the Role of Fc Receptors and Complement	- To standardize assays that measure these anti-viral activities
	- To assess their biologic relevance in passive protection experiments in animal models using antibodies that exhibit the different effector functions in vitro
Assay Standardization and Validation	- To standardize and compare neutralizing antibody assays in order to identify the most reliable assay or combination of assays to measure neutralization
	- To use more than one assay to assure that all neutralizing antibodies are detected
	- To prioritize standardization of the PBMC assay because it is the only one partially validated in passive antibody experiments in animal models
	- To validate neutralization assays based on new technologies in passive antibody experiments in animal models
	- To generate new SHIVs from nonclade B viruses to address the above issues
Immunoregulation of B-cell Responses	- Better understanding of B-cell responses regulation (and dysregulation) is needed in order to identify the best way to induce long-lasting neutralizing antibodies
	- To identify genes that are associated with the wide variation in neutralizing antibody responses in HIV-1-infected individuals and in vaccine recipients
	- To study in nonhuman primate models the potential functional contributions of B-cells to HIV infections
	- To establish a research consortium to study fundamental B-cell biology as it relates to HIV-1 vaccines

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do not need to be neutralizing, (ii) effector cells belong to native immunity and are therefore ready to go, (iii) ADCC does not attack the virus, but clears infected cells displaying viral antigens, a mechanism reminescent of T-cell immunity. However, unlike CTLs, ADCC does not distinguish infected cells from uninfected ones on which viral particles or antigens are absorbed (for example, soluble gp120 on membrane CD4). Despite this limitation, it is conceivable that a preventive vaccine inducing high titers of Abs mediating ADCC should be effective at curbing viral replication at the very beginning, tipping the balance in the virus—host dynamics in favor of the host.

Although these recent advancements have spurred new interest in the feasibility of inducing protecting Ab responses, many vaccine approaches, such as those based on DNA and viral vectors, have recently focused on the induction of cellular immune responses.[6,126-128] The rationale is that a cellular immune response against HIV, although unable to provide sterilizing immunity, should hopefully enable vaccines to control virus replication following infection, contain viral load, slow down progression toward disease and reduce the probability of secondary infections.[129]

Long-lived memory virus-specific T-cell responses have been shown to be critical to the control of viral replication in many chronic infections including cytomegalovirus, Epstein-Barr virus, human papilloma virus, hepatitis C virus and also HIV (for a review see ref. 29). Despite a large body of evidence suggesting that CTLs,[130,131] T-helper cells[130,131] and Tregs[91] play an important role in controlling infection, direct proof is still lacking. Further, in contrast to Ab responses, T-cell responses are technically more difficult to investigate. In fact, in most vaccine studies IFN-γ ELISpot is used to evaluate CTLs because of its ease, robustness, reproducibility and sensitivity.[132-134] However, while this assay is suitable to measure vaccine immunogenicity, IFN-γ production by CTLs alone did not correlate with control of infection[135,136] (for a review see ref. 137). Similarly, multimer staining technology has provided powerfully insights into dynamics of epitope specific CTLs and their pressure to select escape viral variants. However, this technique requires knowledge of MHC haplotypes and at present it can be applied to a limited number of specificities, making it unsuitable to monitoring of the global T-cell response.

More recent advancements in the measurement of T-cell responses demonstrated that polyfunctional $CD4^+$ and $CD8^+$ T-cell responses are a much better correlate of control of infection or survival. Prolonged (4 years) control of SHIV-89.6P infection in macaques primed with DNA and boosted with modified vaccinia virus Ankara (MVA), both encoding Gag, Pol and Env of SHIV-89.6P, correlated with low breadth and frequency of polyfunctional (IFN-γ and IL-2) $CD4^+$and $CD8^+$ T-cells.[138] The low breadth and frequency of these responses might reflect the limited antigenic stimulation due to the strong suppression of virus replication. However, it might also be due to containment of the detrimental immunoactivation observed in pathogenic HIV and SIV infections, since naturally SIV-infected sooty mangabeys, which have a high viral load but limited immune activation, develop a similar pattern of $CD8^+$ T-cell responses.[139] In a SIV model, the prolonged survival of rhesus macaques vaccinated with plasmid DNA followed by boosting with replication defective adenoviral vectors encoding SIV Gag, Pol and Env and challenged with SIVmac251 correlated with the magnitude and preservation of Gag-specific polyfunctional central memory $CD4^+$ and $CD8^+$ T-cells, while set-point viral load was not predictive.[96,98] In a similar study, preservation of polyfunctional $CD4^+$ T-cells during the first two weeks of infection was a strong predictor of prolonged survival and it was associated to the rapid appearance of Abs neutralizing the challenge virus, which may have contributed to the significant reduction of set point viral load observed in vaccinated animals.[140] Intriguingly, this prolonged survival occurred despite vaccinated animals underwent a substantial, although not massive, $CD4^+$ T-cell depletion in the gut, suggesting that CD4 loss in the gut is not invariably associated to progression, as indicated by the studies in natural hosts.[92,94] Taken together these data suggest that preservation of virus-specific polyfunctional T-cells is an important predictor of control or prolonged survival. The differences observed in these studies may be due to the different vaccine strategies, which may have different correlates of protection, as well as to the virus type and dose chosen for the challenge (for a review see ref. 14). The relevance of the challenge virus is suggested by the monovalent Ad5-gag vaccine developed by Merck (MRKAd5), which was shown to elicit a potent CTL response in rhesus

monkeys and protect from disease progression upon intravenous challenge with the pathogenic SHIV-89.6P,[141,142] but to a much lesser extent against an intrarectal challenge with SIVmac239.[143] Notably, the limited protection upon SIVmac239 occurred in the presence of T-cell responses that correlated with protection in the former study.[144] Thus, the recent failure (no reduced transmission or viral load in vaccinees without pre-existing immunity to the vector) of the Phase II clinical trial based on the MRKAd5 trivalent vaccine would suggest SIV as a more rigorous challenge virus and better predictor of vaccine efficacy in human.[145,146] An additional factor complicating the identification of correlates of protections is the possibility that T-cell responses effective at controlling progression in LTNPs, when induced by vaccination prior to infection may actually favour the selection of escape mutants and accelerate progression.[147] Such an acceleration may also occur because of the expansion of HIV-specific $CD4^+$ T-cells induced by vaccination, which actually increases the number of susceptible target cells to the incoming virus.[148] Thus, despite major advancements in the field, solid correlates of protection are still lacking. The recent introduction in the HIV vaccine field of midsize Phase IIb test of concept (TOC) trials basically acknowledges this weakness and is aimed at obtaining valuable information about vaccine efficacy before moving to much larger, longer and costly Phase III efficacy trials.

General Strategies Adopted to Induce Protective Immunity

Vaccines Aimed at Inducing Neutralizing Abs: Vaccines Based on HIV-1 Env Protein

Former subunit vaccine candidates were mainly focused on the use of the structural protein Env as the immunogen aimed at blocking virus adsorption to the target cells by inducing broadly NAbs.[99,149] However, vaccination with the monomeric HIV Env subunit (gp120), elicited Abs that were able to neutralize lab-adapted but not primary virus isolates[150] and homologous but not heterologous viruses in preclinical challenge models.[151,152] Thus, despite numerous attempts, these approaches have failed so far in eliciting durable, cross-clade NAbs needed to achieve sterilising immunity, as recently soberly confirmed by the failure of the first two Phase III clinical trials of gp120 envelope subunits, AIDSVAX B/B and AIDSVAX B/E by VaxGen, tested in over 5,000 at-risk volunteers in the United States and in Thailand, respectively.[153-156] The reason for this failure was likely related to the complex structure of Env and its high variability.[157-159] Further, heavy glycosylation of the gp120 molecule creates a glycan shield, protecting the protein from incoming NAbs, a phenomenon unknown at the time of the first Env immunizations.[160] In fact, Env hides its Ab binding sites under the protein loops and the heavily glycosylated sites on its surface, hampering recognition of relevant, mostly conformational, epitopes by NAbs.[161-163] Despite the tremendous effort and the sobering results, HIV-1 Env remains a key target for new vaccine strategies (Table 2). In the recent years much effort has been devoted to the construction of oligomeric (trimeric) forms of Env which closely mimic the structure of the native protein present on the viral envelope[164,165] and have been shown to be superior at inducing Abs directed towards conformational epitopes[166,167] and capable of neutralizing both T-cell-line adapted (X4) and selected (R5 and X4) primary isolates of HIV-1,[168,169] as compared to the monomeric gp120.[170] However, Env trimers are extremely unstable and several approaches have been undertaken to stabilize them. In one approach, the gp120-gp41 cleavage site was disrupted by mutagenesis, generating an uncleaved form of gp140 ($gp140_{UNC}$).[171-176] Some of these uncleaved forms of the Env proteins were moderately superior to monomeric gp120 for induction of NAbs in small animal models.[167,177-180]

In another approach, an intermolecular disulfide bond between gp120 and gp41 (SOS gp140) was introduced, with an expectation to induce both neutralizing and fusion-blocking Abs.[181-183] Priming with DNA encoding a membrane-bound form of the SOS gp140 protein followed by repeated immunization with the soluble trimers resulted in high titer Abs that neutralized neutralization sensitive lab strains and, to a much lesser extent, primary heterologous HIV-1 strains.[184] Since the gp41-gp41 interactions in SOS gp140 were too weak to maintain the protein in a trimeric configuration,[183] a single residue change, I559P, within gp41 was introduced.[182] This variant of

SOS gp140, designated SOSIP gp140, appear to be fully cleaved, to be predominantly trimeric and to have favourable antigenic properties.[165] In a recent study, comparison of the immunogenicity of SOSIP gp140 trimers with uncleaved gp140 trimers and monomeric gp120 using a DNA prime-protein boost immunization regimen in rabbits, indicated that SOSIP gp140 trimers were superior to $gp140_{UNC}$ and gp120 proteins at inducing NAbs.[170,184] and SOSIP gp140 trimers with Env from other clades have been generated, which have comparable or better stability and capability to induce NAbs as compared to a prototypic strain (JR-FL, subtype B).[164,185,186]

In a third approach, heterologous trimerization domains at the terminus of the gp41-ectodomain[187,188] were introduced to stabilize the molecule and 30 aminoacids in the second hypervariable region (V2) were deleted to expose neutralizing epitopes shielded by V2.[189,190] This particular variant, termed ΔV2 Env, has been developed and tested in preclinical models including rabbits and monkeys and is currently being evaluated in a gag + env DNA/PLG prime-ΔV2 Env protein boost preventive Phase I trial.[168,169,177,189-191]

Since critical neutralizing epitopes are displayed very transiently upon binding to CD4, another strategy developed by Merck and by the University of Maryland[192] is based on covalently linked monomeric gp120 or oligomeric gp140 to soluble CD4 or to synthetic mimetics of the CD4 receptor in order to induce the conformational changes that take place upon binding of the virus to CD4 prior to virus entry, thus revealing critical neutralizing epitopes, such as those involved in coreceptor binding. This approach was tested in macaques and shown to elicit broadly cross-reactive NAbs.[193] Finally, broadly NAbs are presently being exploited to select peptides from phage-display libraries that mimics the Env neutralizing epitopes (mimotopes) with the goal of identifying immunogens capable to elicit broadly NAbs (for a review see ref. 105).

Vaccines Aimed at Inducing Cellular Immunity: Vaccines Based on Gag, Pol or Nonstructural HIV-1 Proteins: Rev, Tat and Nef

Due to the obstacles encountered in the preparation of an anti-Env vaccine providing sterilising immunity and the protective role of cellular immunity (see correlates of immunity section), a second generation of vaccines based on the structural protein Gag has been developed, with the concept of inducing strong and broad T-helper and CTL responses, which, would contain virus replication, thereby protecting from disease progression and reducing virus transmission to healthy individuals. In fact, Gag protein seems to be the most potent of all HIV-1 antigens in eliciting CTLs, it is more conserved in its immunodominant epitopes than Env and, most importantly, the breadth of CTLs to Gag but not to other HIV proteins appears to correlate with nonprogression in a large cohort study conducted in South Africa on untreated individuals.[63] Ongoing trials with the Gag antigen show promising induction of cellular immunity in primates and humans (for a review see ref. 194).

A Gag-Pol DNA vaccine has been recently tested and resulted safe and well tolerated. However, no HIV-specific Ab responses and only low-magnitude HIV-specific T-cell responses were detected.[195] Further, evidence from preclinical testing of a Gag/Env DNA vaccine in monkeys indicate that initial control (i.e., undetectable plasma viremia level) against challenge with the pathogenic virus SHIV89.6P was overcome over time by the appearance of escape mutants despite apparently preserved anti-viral humoral and cellular responses.[196,197] Alternative strategies have been recently considered, based on the new concept of "reverse vaccinology" with the aim of blocking virus replication and disease onset by targeting nonstructural HIV regulatory genes such as Tat, Rev and Nef, which are essential for replication and infectivity.[153]

In particular, these proteins share desirable features for the generation of a promising vaccine since they exert key functions in the early virus life cycle, contribute importantly to infectivity and pathogenicity, induce a broad immunity and they are highly conserved in their immunogenic domains across HIV-1 clades[198,199] (Table 3). In fact, these proteins are produced very early after infection, Tat and Nef even before HIV integration in quiescent T-cells in which they promote cellular activation and viral replication.[200] Emerging data indicate that, despite their small size, regulatory and accessory proteins are targeted by cellular immune responses very early in the course

Table 3. Rationale to use Rev, Tat, and Nef for an HIV/AIDS vaccine

Evidence	Description	Reference
Pathogenetic	Rev, Tat and Nef are expressed very early and strongly dysregulate the immune system contributing importantly to the establishment of infection and to disease progression	15,200,205,241,251
Epidemiological	In asymptomatic individuals responses to these nonstructural proteins significantly correlated with non-progression to disease	74,201,225,229,481
Immunological	Rev, Tat and Nef are conserved in their functional and immunogenic regions (both B- and T-cell epitopes). Further, Tat and Nef display immunomodulatory effects on APCs exerting adjuvant effects and determining the type of immune response elicited	198,212,215,216,482
Preclinical	Tat, Rev and Nef, either alone or in combination, have demonstrated in preclinical models to be safe and to elicit broad and specific immune responses and, more importantly, to control viral replication and to block disease progression	For a review, see refs. 240,483
Clinical	Tat, Rev and Nef either alone or in combination have demonstrated in Phase I trials to be safe and to elicit broad and specific immune responses	For a review, see ref. 240

of natural HIV-1 infection and contribute importantly to the total HIV-1-specific $CD8^+$ T-cell responses, since multiple CTL epitopes have been identified in functionally important regions of these proteins.[201-204] Furthermore, they have immuno(dys)regulatory effects aimed at facilitating target cell recruitment and activation, further promoting HIV replication and spreading.[205-207] Of note, Tat and Nef are also found extracellularly and in this form they exert effects on different cell types, including chemotactic activity for HIV target cells.[208]

In particular, extracellular Tat can enter both infected and uninfected cells, where it promotes HIV replication or modulates the expression of cellular genes, respectively (for a review see ref. 209). Among others, extracellular Tat upregulates the expression of chemokine receptors and HIV coreceptors, CCR5 and CXCR4.[210,211] Extracellular Tat has also important effects on immunoregulatory functions (for a review see ref. 209) (Table 3). In particular, bioactive soluble Tat selectively binds and enters both immature and mature DCs (iDCs and mDCs, respectively), drives iDCs maturation and activation toward a T-helper 1 (Th-1) inducing phenotype,[212] gains access to the MHC class I pathway of presentation,[213,214] and modulates the proteasome catalytic subunit composition, modifying the hierarchy of the CTL epitopes presented in favor of subdominant and cryptic epitopes.[215,216] This latter activity might be of relevance since one way used by HIV-1 to escape CTL recognition is to mutate residues in the epitope that prevent or impair processing and presentation.[217,218] Accordingly, in the majority of the multiply exposed uninfected sex workers of the Nairobi cohort, CTLs recognize epitopes that are either subdominant or not recognized in infected women.[24] It remains to be seen whether Tat contributes to it in the course of natural infection, whether targeting Tat impacts on this type of immune evasion, or whether this property of Tat may be exploited to induce broader T-cell responses by including it in HIV/AIDS vaccines targeting other antigens. Indeed, preliminary data indicate that in mice co-immunization with Gag and Tat induces CTL responses against 11 different T-cell epitopes, as compared to mice vaccinated with Gag alone, which only responded to 6 epitopes.[219] Both cellular and humoral Tat-specific immunity may contribute to the control of infection and/or disease progression. Because HIV-infected cells express Tat very early after infection, vaccine-induced anti-Tat CTLs may eliminate infected cells and block HIV infection at an early stage.[220] In fact, rapid induction of anti-Tat CTLs has been reported in naïve rhesus macaques acutely infected with the pathogenic SIVmac239 molecular clone, leading to the selection of apparently less aggressive virus variants.[221] Notably, anti-Tat CTLs were found more effective than anti-Gag CTLs at suppressing virus replication in Mamu-A*01 rhesus macaques.[222] Of outmost importance, these data have been recently confirmed in patients enrolled prior to seroconversion and in which a strong temporal correlation between anti-Tat CTLs appearance (as early as 8 days postinfection), viral load decline and $CD4^+$ T-cells recovery was found.[74]

Consistent with this hypothesis, the presence of Tat-specific CTL responses correlates with nonprogression to AIDS both in SIV-infected monkeys and in HIV-positive individuals.[223-226] Furthermore, anti-Tat Abs can sequester the extracellular protein, thus preventing the extracellular Tat-driven enhancement of infection and immune dysregulation associated with them. Strikingly, anti-Tat Abs, which are found only in a minority (15-20%) of HIV-1 infected individuals, are almost exclusively present during the asymptomatic phase of infection and correlate with nonprogression to AIDS.[227-231] Whether this indicates that neutralization of extracellular Tat may impact on disease progression or is merely a reflection of an underlying effective and broad immune response is currently under investigation. Tat is also highly conserved in its immunodominant domains, as suggested by the observation that sera from Ugandan and South African individuals infected with nonclade B HIV-1 strains cross-react with the Tat protein of an HIV-1 clade B strain.[198]

Vaccines based on HIV-1 Tat (both protein and DNA) have proven to be safe and immunogenic in mice and protective in monkeys.[232-236] However, these results have not been confirmed by other investigators utilizing different Tat formulations and vaccination strategies.[237-239] Whether these apparently conflicting results are due to the nature of the vaccine antigen (DNA, native versus inactivated Tat protein, vectored antigen), the monkey species, the route of the administration,

the antigen dose and schedule of immunization, the adjuvant used, or the virus challenge dose, still remains to be elucidated.[240]

Rev is also absolutely required for HIV replication since it facilitates the nuclear export of intron containing viral mRNAs allowing the transition from the early to the late phase of gene expression and proviruses lacking Rev do not produce virions.[241] While it is presently unknown whether Rev is released extracellularly and exerts effects on neighbouring cells, Rev, like Tat and Nef, is often targeted by CTLs in HIV-positive individuals[201] and is broadly conserved among different HIV-1 clades in its functionally constrained and immunodominant domains at the N terminus.[242] However, spontaneously occurring mutations in Rev reduce HIV-1 structural gene expression to levels undetectable by CTLs and may represent a mechanism to escape immune recognition.[205] Of importance, HIV-1 Tat and Rev are the dominant viral proteins produced before Nef down-regulates MHC class I molecules on the cell surface, hampering recognition of infected cells by CTLs[65] (Table 3). Vaccine based on Rev and Tat has proven protective in monkeys.[243] Rev alone or in association with Nef and Tat has been used for therapeutic vaccination and resulted to be safe and immunogenic in HIV-1 infected individuals.[244-246]

HIV-1 Nef protein is a myristoylated, membrane-associated cytoplasmic protein abundantly expressed in the early phase of HIV-1 replication and released in the extracellular milieu.[247,248] Nef protein serves multiple functions and is likely to contribute to viral pathogenesis by downregulating CD4 and MHC class I on the surface of infected cells[249] (Table 3). Among its biological activities, Nef mediates receptor down-regulation, T-cell activation and cytoskeleton rearrangement.[250,251] The Nef protein also enters B-cells and suppresses immunoglobulin class-switch contributing to the evasion of protective T-cell-dependent IgG and IgA responses.[252] In addition, Nef interferes with the ability of CTLs to kill infected T-cells by decreasing the surface expression of MHC class I on HIV-1-infected T-cells[65] and favours spreading of HIV to T-cells by increasing the expression of DC-SIGN on DCs, which traps infectious HIV particles[253] and by promoting DC maturation.[254] Further, differently from Tat, Nef induces FasL upregulation and apoptosis in bystander cells both in vitro and in vivo,[255] whereas it selectively spares infected cells,[256] contributing to immune evasion and pathogenesis. Finally, infection of MDDCs with a wild type but not with a nef defective HIV-1 induces the release of soluble factors recruiting and activating lymphocytes, which consequently become targets for productive HIV infection.[257]Taken together these data strongly support the view of Nef as an important vaccine candidate alone or in association with other HIV antigens (for a review see ref. 240). Immunization with plasmid DNA or a MVA vector expressing Nef was demonstrated to be safe and immunogenic in preclinical and clinical studies.[258-260]

Vaccines Combining Structural and Nonstructural HIV-1 Gene Products

Experimental evidence on the role played by regulatory and structural HIV gene products in HIV infection and pathogenesis represents the rationale to develop new vaccination strategies based on the combination of the two classes of proteins (Table 4). These strategies range from a "minimalistic" approach in which only two antigens, one regulatory (Tat or Nef) and one structural (ΔV2-Env) HIV protein are combined, to a "maximalistic" one, which is aimed at imitating a live attenuated vaccine and therefore combines many HIV structural and nonstructural genes. Several of these new generation vaccines are currently under evaluation in preclinical and clinical trials in the context of the European AIDS Vaccine Integrated Project (AVIP) (http://www.avip-eu.org).[261-266] Such combined vaccines should be able to generate immune responses to both viral products, which are expressed early (regulatory proteins) or late (structural proteins) during the viral life cycle, thus maximizing immune targeting of viral infection. The criteria for an advancement of any of these combined vaccines towards Phase II/III trials in Developing Countries are safety and the demonstration of stronger and broader immune responses against each antigen, compared to those elicited upon immunization with each antigen separately.

In particular, the combination of the regulatory HIV-1 protein Tat with the structural protein ΔV2-Env, represents one of the most recently developed HIV vaccine candidates.[261] Preclinical studies in mice and monkeys have shown that the Tat/ΔV2-Env vaccine is safe, immunogenic and

Table 4. Combined vaccine candidates to be tested in Phase I trials within the AVIP Consortium: previous work and development stage of single components

Combined Vaccine Candidates	Single Components	Mice Immunogenicity	Mice Efficacy[1]	Monkey Efficacy	GMP Development	Approval for Human Use	Clinical Trials (Performed or Ongoing)[2]
Tat + ΔV2Env[3] (clade B)	Tat	+	-	+	+	+	Completed (P + T)
	ΔV2Env (clade B)	+	-	+	+	Pending	To be started (P)
Nef + ΔV2Env (clade B)	MVA[4]-Nef	+	+	+	+	+	Completed (T)
	ΔV2Env (clade B)	+	-	+	+	Pending	To be started (P)
Nef, Rev, Tat, Gag, RT, Env	Nef, Rev, Tat	+	+	+	+	+	Completed (T)
	Gag, RT, Env	+	+	-	+	+	To be started
Multi HIV B-clade Ags and epitopes[5]	Multigene[6]	+	+	-	+	+	Completed (P + T)
Multi HIV A-clade Ags and epitopes[5]	Multigene	+	+	-	+	Pending	To be started
Multi HIV C-clade Ags and epitopes[5]	Multigene	+	+	-	+	Pending	To be started
Multi HIV FGH-clade Ags and epitopes[5]	Multigene	+	+	-	+	Pending	To be started

[1]Mice efficacy is evaluated as the capability of HLA-A2 transgenic C57Bl/6 mice to eliminate the engraftment of HIV-1/MuLV-infected syngeneic splenocytes injected intraperitonally;[484] [2]P: Preventive; T: Therapeutic; [3]ΔV2Env: Env deleted in V2 (see text for further details); [4]MVA: Modified Vaccinia Ankara virus; [5]Multi HIV, also termed MultiHIV DNA vaccine is a plasmid expressing an antigenic fusion protein composed of the regulatory HIV-1 proteins Rev, Nef and Tat, Gag p17/p24 and a stretch of 11 cytotoxic T-lymphocyte (CTL) epitope clusters from Pol and Env, which was cloned into a novel DNA vector named the Gene Transport Unit (GTU). Four different plasmids expressing the same immunogens but originating from subtypes A, B, C consensus, or FGH ancestral sequences, are currently under evaluation; [6]Multigene is a cocktail of seven plasmids encoding clade B Nef, Rev, Tat, RT, clade A and B Gag and clade A, B and C Env proteins.

protects monkeys against an intrarectal challenge with $SHIV_{SF162p4}$ (ref. 267 and Ensoli B, unpublished data) or an intravenous challenge with SHIV89.6P.[268] Based on these promising results, a Phase I clinical trial will start in 2008. The minimum criterium of success for Env-specific responses will be the induction of NAbs against the vaccine strain (i.e., homologous neutralization) in at least 50% of the trial participants.

The other minimalistic approach developed within the AVIP consortium is based on the combination of Nef and ΔV2-Env[264] (Table 4). This vaccine is composed of the *nef* gene inserted into the MVA (see below) in combination with the same ΔV2-Env protein mentioned before. As for the Tat/ΔV2-Env approach, the Nef/ΔV2-Env vaccine will be administered in HIV-negative volunteers, seeking to induce mostly anti-Nef cellular immunity and anti-Env humoral immunity, in particular NAbs, to prevent (or reduce) virus entry and to control virus replication. As for the Tat/ΔV2 Env vaccine, criteria for advancement of the Nef/ΔV2 Env vaccine beyond Phase I will be proven safety and broader and more potent immune responses against the components of the combined vaccine, as compared to those obtained upon vaccination with the single antigens. Phase I trials with Nef or ΔV2-Env alone have been completed[189,259] preclinical testing in macaques of the Nef/ΔV2-Env combined vaccine will be carried out in 2008 and preventive Phase I studies will follow shortly.

The multigene strategy represents another attractive vaccine approach based on the design of a cocktail of genetic immunogens (DNA constructs) encoding several viral components from various subtypes of HIV-1, including structural and regulatory proteins as well as viral enzymes.[266] Two multigene approaches are being evaluated in the AVIP Consortium, one containing a cocktail of seven plasmids encoding Nef, Rev, Tat, Gag, RT and Env antigens, termed HIV multigene;[266] the second one, based on the genes coding for Rev, Tat, Nef, Gag, p17, p24 full length antigens, also includes over 20 T-cell epitopes from Pol, Protease and Env antigens and is therefore termed Multi-HIV antigens/epitopes.[265] A prophylactic Phase I trial with the HIV multigene was recently conducted in Sweden and shown to be safe and highly immunogenic. More than 90% of the 38 volunteers mounted T-cell responses (proliferative and IFN-γ ELISpot responses) against the vaccine antigens upon administration with a needle-free device (Biojector) of DNA encoding Env (clade A, B, C), Rev (B), Gag (p17/p24, A and B), RT (mutated, B) followed by a boost with MVA expressing Env, Gag and Pol of CRF01A_E.[269] Based on these encouraging results showing that this administration strategy and the use of GM-CSF as adjuvant increases the immunogenicity of a DNA prime followed by MVA boosting in human, a new preventive Phase I/II study started in 2006 in Tanzania.[270] As part of the AVIP program, a therapeutic Phase I-II trial has recently started in UK and Sweden with the aim of comparing in individuals infected with HIV clade B the immunogenicity of the multigene vaccine based on clade B antigens with that of the clade A-C multigene vaccine. Of note, both vaccines include a newly developed plasmid encoding Tat/Nef as a single fusion protein.[266] The Multi-HIV antigens/epitopes approach exploits a novel delivery system termed gene transport unit (GTU), a proprietary technology of FIT BIOTECH, which increases the immunogenicity of DNA vaccines thereby avoiding the need for an heterologous boosting.[265] Phase I studies carried out in Finland have proven that GTU-MultiHIV (B clade) is safe and immunogenic in healthy and HIV-1 infected individuals and it is currently being tested in a therapeutic Phase IIa clinical trial in South Africa.[265] A second generation vector, called Auxo-GTU, was more recently prepared and a constructs expressing multiple antigens and epitopes from several clades (A, B, C, FGH) was made and it is going to be evaluated in a Phase I/II trial in 2008.

To address the issue of viral diversity, a novel strategy to broaden cross-clade T-cell responses has been recently proposed. According to this strategy, polyvalent vaccines can be made that comprise a mosaic of several naturally occurring sequences computationally optimized to include the maximum number of potential T-cell epitopes from relatively conserved and immunologically relevant HIV-1 proteins.[271] In vivo testing will verify the validity of this approach.

Key Issues Relevant to HIV Vaccine Development: How to Get the Right Responses in the Right Places

Mucosal Vaccines

Since most of the HIV-1 infections are caused by mucosal transmission both horizontally (sexual intercourse) and vertically (child delivery and breast-feeding), an AIDS vaccine must primarily elicit a robust immune response at the mucosal surfaces.[110] Most of the AIDS vaccine candidates that are currently in clinical trials around the world are delivered by intramuscular or intradermal injection. These routes of administration induce Ab and cellular immune responses in peripheral lymphoid tissues (systemic immunity), although evidence in human of mucosal responses upon intramuscular vaccination with a recombinant canarypox HIV-1 vaccine have also been reported.[272] Furthermore, studies of SIV infection in macaques indicated that, regardless of the route of infection, the gastrointestinal and vaginal mucosae represent the major site of virus replication and amplification and the initial sites of $CD4^+$ T-cell depletion.[273-275] In particular a rapid depletion of $CD4^+$ T-cells has been observed in the vagina of SIV-infected macaques, particularly among the $CCR5^+$ $CD4^+$ subset that is the preferential target for elimination by SIV infection.[276]

Several studies indicated that secretory IgA inhibit virus assembly and intracellular release and play an important role in inhibiting HIV transmission via the mucosal route, in multiply exposed females.[277,278] Further, multiple rectal exposures to low doses of SIV induced MHC class I-restricted cytotoxic responses that protected against a mucosal challenge with an heterologous virus.[279] Similarly, studies in the macaque model provided insights on the protective role of high-avidity mucosal CTL responses generated upon intrarectal vaccination.[280,281] Taken together these data suggest an important protective role for IgA and CTLs at the portal of virus entry. In this regard, mucosal immunisation appears to be more effective than systemic vaccination at eliciting humoral (IgA and IgG) and cellular ($CD8^+$ CTLs) immune responses.[282,283] However, not all the mucosal routes of vaccine administration are equally good at inducing immune responses at the different mucosal surfaces in the body. For instance, oral vaccines are effective at preventing infections that primarily target intestinal tissues, but are not very efficient at inducing IgA in the vagina, one of the main ports of entry of HIV-1.[284] A few studies have suggested that local administration of protein vaccines to the mucosa of the genitourinary tract induces a weak to modest local immunity at the site of immunization.[283,285] In contrast, intranasal (IN) immunization has been shown to induce local immunity not only in the nasal-associated lymphoid tissue and lung, but also in the female genital tract in rodents,[286,287] human[288] and nonhuman primates.[289] Of note, it has been recently reported in the mouse that the nasal associated lymphoid tissue is also an inductive site.[290] Taken together, these data make this type of immunization, which is more practical than vaginal vaccination, appealing to AIDS vaccine researchers. In this regard, immunization of HIV-1 seronegative women with an Env subunit vaccine administered either intranasally or intravaginally, together with a mucosal adjuvant shown to be effective in mice, failed to induce detectable IgA or IgG.[291] These negative results should not stop the attemps to use the IN route. In fact, various protein-, DNA- and RNA-based immunopotentiating adjuvants/delivery systems as well as of live bacterial and viral vectors, which may differ in their ability to induce a specific type of immune response (e.g., CTLs versus antibody responses) at the desired site,[292] are available for IN immunisation and should be evaluated. However, since the nose is a well-known port of entry for neurotropic viruses, there are safety issues that will need to be fully addressed before testing IN vaccines in clinical trials. Of note, tonsillar immunization with an attenuated SIV (SIVΔNef) induced systemic immune responses and conferred protection upon intrarectal challenge of rhesus macaques with a pathogenic SIV.[293] It will be of interest to determine whether safer vaccine approaches will afford similar protection. Recent reports have suggested that combinations of mucosal and systemic immunizations may enhance both mucosal and systemic immune responses.[294-297]

Mucosal tissues are rich in antigen presenting cells (APCs), specialised immune cells that are involved in the induction and regulation of anti-viral immunity. DCs represent the most potent APCs for naïve T-lymphocytes and they are among the first cells in the body to come in contact

with HIV.[298] These cells are critical in the early phases of infection, working as sentinels, alerting the immune system and controlling its early decisions. Furthermore, they exert a crucial role in the induction and regulation of adaptive immune responses.[299] Therefore, it is critical to design vaccine formulations capable of properly targeting and stimulating DCs to induce strong immune responses at mucosal sites. It is likely that the stimuli received by DCs in the peripheral compartments affect their ability to activate T-cells and/or B-cells as well as the type of T-cell response elicited.[300] Of interest, in the mouse, a single intracolorectal administration of a replication defective adenoviral vector expressing OVA or Herpes simplex virus (HSV)-2 glycoprotein B antigen targeted mucosal DCs, which migrated to the draining lymph nodes and induced adaptive immune responses at the rectal and vaginal level that protected the animals against a challenge (by either route) with vaccinia expressing OVA or a lethal dose of HSV-2, respectively.[301] Similar protection was not afforded by IN, intravaginal, or subcute vaccination, suggesting that adenoviral vectors, which naturally target DCs, may be particularly suitable to induce protective humoral and cellular immune responses at sites that represent the major portals of entry of HIV. It remains to be seen whether these promising results are confirmed in human. In fact, intramuscular immunization with replication-defective Ad5 vectors expressing HIV-1 gag, pol, or nef failed to reduce transmission or lower viral load in high risk individuals that became infected.[146] In this regard, manipulation of antigen presenting cells to elicit virus-specific cellular responses is a promising tool to control persistant viral infections.[302-309] In fact, studies in monkey[310] and human[311] indicate that inactivated whole virus-pulsed DC vaccines may be an effective strategy for treating people with chronic HIV-1 infection.

Delivery Systems

Subunit (proteins or peptides) vaccines are generally very safe, with well-defined components. However, these antigens are often poorly immunogenic and adjuvants are required to induce a measurable and supposedly adequate immunity. Thus, a vast array of delivery systems (e.g., micro/nanoparticles, emulsions, ISCOMS, liposomes, virosomes and virus-like particles), immunomodulators (cytokines, chemokines or costimulatory molecules) and, as mentioned above, even autologous DCs pulsed with viral antigens have been proposed and are presently being used to increase the efficiency of vaccines against HIV/AIDS (Table 5). Furthermore, several highly attenuated replicating and nonreplicating vectors have been or are being tested in a number of preclinical and clinical trials (ref. 240 and Table 6)

These strategies, reviewed elsewhere,[240] have proven effective in controlling viremia and progression to AIDS in nonhuman primates, but observations in early phase clinical trials in humans have not been promising. In fact, some of the trials had to be stopped at various stages due to adverse reactions to the delivering vector[312] or the inability of the expressed immunogen to cover genetically diverse isolates prevalent in the geographical areas.[313] Nevertheless, the outcome of several ongoing clinical trials is expected to deliver good news about safe vaccine delivery vectors and, if possible, an effective vaccine against a particular strain of HIV-1[9] (Table 6). Here we briefly review the different approaches utilized to deliver vaccines that are currently being evaluated in clinical trials (Table 5).

Plasmid DNA

Genetic vaccines (naked-DNA vaccines), employ DNA plasmids as "Trojan horse" vectors to deliver genes that code for HIV epitopes (for a review see ref. 314). These expression vectors remain in their episomal form into the host cell where they produce peptides that induce cellular immunity. Compared to viral and bacterial vectors, DNA plasmids focus the immune response on more narrowly on HIV insert sequences, do not induce (and are not affected by pre-existing) immunity to the vector, are cheap and have several regulatory, safety, handling advantages.[314] Immunization with DNA plasmids containing HIV inserts has been demonstrated to elicit substantial cellular response in mice and nonhuman primates,[315,316] but not in humans.[317]

Therefore, many strategies have been undertaken to enhance the immunogenicity of genetic vaccines, which include delivery systems, modifications of the vaccine construct, formulation with immunostimulatory molecules.[314] In particular promoter modification and inclusion of genes

Table 5. Major HIV-vaccine approaches

Types of HIV Vaccine	Advantages	Disadvantages	Type of Response Elicited
Whole HIV Viruses			
- Killed/Inactivated Viruses	Simple to prepare; they might present HIV surface proteins in a relatively native conformation depending on the inactivation procedure; no mutation or reversion	Little efficacy in nonhuman primates; safety concerns (inactivation efficiency)	Few NAbs, no CTL response
- Live Attenuated Viruses	Mimic natural infection; high levels of protection in animal models	Safety concerns: mutation; potential reversion to virulence	Long-lasting cell-mediated and humoral immunity
Recombinant Viral Proteins (Subunit Vaccines)	Safe; simple and inexpensive to prepare; defined composition (mostly structural proteins)	Immunogenic response is restricted to selected antigens; responses are not durable; no protection in two efficacy trials; adjuvant required	Target humoral immunity, no CTL response
Peptides	These vaccines use small pieces of HIV proteins as an immunogen; safe, inexpensive, potentially useful for broad antigenic diversity	Poorly immunogenic in human trials; stability issue	Poorly immunogenic in human trials; adjuvant required
Naked DNA	Safe; stable; no cold chain required; inexpensive; potential to encode multiple antigens; immunogenic in animals; prolonged immunity; more effective in heterologous prime-boost strategies	Poorly immunogenic in humans; concerns about DNA integration into human cells	Cellular immune responses; heterologous prime-boost strategies needed to induce humoral responses in primates
Viral Vectors	The vaccine is a weakened virus, unrelated to HIV, into which HIV genes are inserted; high-level production of protein antigens directly within cells of the immunized host, potential adjuvant effects of the viral delivery system itself; delivery of antigen directly to components of the immune system, such as antigen-presenting cells	Complicated to prepare; viral escape mutants; potential immunodysregulatory effect of the vector proteins; pre-existing immunity	Depending on the vector, systemic and mucosal humoral and cell-mediated immune responses

continued on next page

Table 5. Continued

Types of HIV Vaccine	Advantages	Disadvantages	Type of Response Elicited
- Poxviruses	Highly immunogenic¸ they grow to high titers, are very stable when lyophilized and are capable of accepting large transgene sequences; potential to be administered by different routes	Safety concerns	Mucosal and systemic antibody and T-cell responses
- ***MVA*** - ***NYVAC***	Safe because they do not replicate in mammalian cells; possibility of introducing large amounts of DNA; effective in nonhuman primate models	Limited immunogenicity and durability of immune responses in humans	Both CTL and Ab responses at both systemic and mucosal sites, depending on the route of immunization
- ***Canarypox (CPV) ex. ALVAC*** - ***Fowlpox (FPV)***	Safe because they do not replicate in mammalian cells; no concerns about pre-existing immunity against these vectors in humans	Limited immunogenicity	T-cell responses
- Adenoviruses (Ad)	Safe (replication incompetent Ad), stable; highly immunogenic; wide tropism; high efficiency of cellular uptake; can be administered at mucosal sites	Responses limited by pre-existing immunity (especially to Ad5)	Robust mucosal and systemic humoral and cellular immune responses, especially when replication-competent Ad vectors are used
- Adeno-Associated Viruses (AAV)	They establish a persistent infection in the host	Poorly immunogenic in human; in mice they induce T-cells with an altered phenotype and functionally impaired	Humoral and cellular immune responses
- Alphaviruses: - ***Sindbis (SIN),*** - ***Venezuelan equine encephalitis (VEE),*** - ***Semliki Forest (SFV) virus***	They can infect a large number of animal cell types and transiently express high amounts of viral and heterologous proteins; safe because their replication occurs exclusively within the host-cell cytoplasm; lack of preexisting immunity; ability to induce apoptosis of transduced cells, favoring DC cross-priming	Anti-vector immune response	Humoral and cellular immune responses

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Table 5. Continued

Types of HIV Vaccine	Advantages	Disadvantages	Type of Response Elicited
- **Herperviruses (HSV)**	Durable immunity; ability to activate the innate immune system; ability to accommodate large inserts of DNA	Safety concerns in immunocompromised individuals; pre-existing immunity	Humoral and cellular immune responses
- **Rhabdoviruses (VSV)**	Rapid growth; relatively safe; mucosal efficiency	Potential safety concerns in immunocompromised	HIV specific CTL response
- **Polioviruses**	They grow to high titers; easy to prepare and to deliver orally; stable in the intestinal tract where they infect the mucosal M-cells responsible for antigenic presentation; immunogenic in nonhuman primates	Pre-existing immunity	Mucosal humoral and cellular immune responses
Bacterial Vectors	Simple and inexpensive to prepare; probably safe; they can be administered at mucosal site	Stability issue	Humoral and cellular responses; mucosal immunity
- **Mycobacterium (BCG)**	Safe in humans; easily administered and affordable; able to induce long-lasting immunity; mucosal delivery of HIV antigens; ability to accommodate large inserts; adjuvant activity	Safety issues especially in children and immuno-compromised hosts	Mucosal and systemic humoral and cellular iimune responses
- **Listeria**	Stimulates innate and adaptive immune responses	Safety issues especially in children and immuno-compromised hosts	$CD4^+$ and $CD8^+$ T-cell responses
- **Salmonella**	Safe; inexpensive; offers the benefit of oral delivery; mucosal targeting	Safety issues especially in children and immuno-compromised hosts; poor immunogenicity in humans	Mucosal and systemic humoral and cellular immune responses
VLPs	Safe; mimic the virus particle, displaying HIV proteins in a relatively native conformation	Difficult to prepare	Good cellular and humoral immune responses at systemic and mucosal level
Microparticles	Enhance the bioavalaibility of the antigen, reducing the number of doses in the immunization schedule; targeting of DCs	Instability and manufacturing difficulties	CTL response

encoding cytokines that increase the expansion of antigen-specific T-cells (IL-2 and IL-15), or attract and induce the maturation of APCs (GM-CSF and B-chemokines) have been evaluated (for a review see refs. 318, 319). Furthermore, novel formulations are being pursued to increase the in vivo expression of DNA vaccines and to protect them from rapid degradation, involving adjuvants or carriers such as liposomes, bacterial endotoxin, macroglobulins, CpG oligodeoxinucleotides, peptides and polymers (described below).[320-322] Nevertheless, DNA vaccines recently tested in clinical trials displayed limited immunogenicity[195,323-325] and most DNA vaccines are presently delivered as a prime in heterologous prime-boosts strategies (see below).

Bacterial Vectors

Among bacterial vectors, live attenuated recombinant *Mycobacterium* spp. and enteric bacteria such as *Salmonella* spp. are microorganisms that can be administered at a mucosal surface and should be able to specifically induce mucosal cellular and humoral immune responses.[326] Bacterial DNA vaccine delivery demonstrated in vivo efficacy in several experimental animal models of infectious diseases.[327] Attenuated strains of *Salmonella* spp. have been developed as potential vectors for stimulating immune responses in the gastrointestinal mucosa.[328] An advantage of these vectors is the possibility to exploit the Type III secretion system, a multicomponent system that allow delivery of antigens directly into the cytoplasm, favouring MHC class I antigen-processing and presentation.[329]

A *Salmonella* in which the SIV gag transgene had been fused to a Type III-secreted bacterial protein was used in a Salmonella-prime/MVA-boost regimen to stimulate SIV Gag-specific CTL responses in the gastrointestinal tract of rhesus macaques.[330] Although low levels of CTLs were detected after the priming, upon MVA boosting strong CTL responses were detected in the blood and in the colonic mucosa. However, no protection against intrarectal challenge with SIV_{mac239} was observed.[330] In a Phase I dose escalation trial oral delivery of *Salmonella* expressing HIV Gag resulted safe and induced strong immune responses to *Salmonella* antigens, but modest immune responses to Gag.[331,332]

Mycobacterium bovis Bacillus Calmette—Guérin (BCG) is another promising vector. BCG has a long record of safety in humans and is able to induce long-lasting immunity.[333] However, despite extensive testing in small animals (mice and guinea pigs), evidence in nonhuman primates of promising immunogenicity[334,335] and efficacy against an homologous challenge after a single inoculation of rBCG expressing the HIV_{MN}V3 loop,[336] recommendations by WHO and UNAIDS to further explore the use of rBCG as a potential vectored vaccine for HIV,[337] no clinical trial has started yet with this vaccine approach. While pre-existing immunity to BCG does not seem to be a problem, use of this vector in developing countries where *Mycobacterium tubercolosis* (and tubercolosis) is highly prevalent and even BCG vaccination may be fatal in immunodeficient children,[338] raise some concerns on the feasibility of large scale vaccination with this platform.

Another interesting vector is represented by *Listeria monocytogenes* (*Lm*), a facultative intracellular bacterium that enters the cell by phagocytosis and colonizes the cytosol of the host cell.[339] Several properties make *Lm* an attractive HIV vaccine vector. First, this bacterium is a good agent to stimulate innate as well as adaptive immune responses since it specifically infects and induces maturation of DCs. Second, foreign antigens encoded by *Lm* are efficiently processed and presented by both MHC class I and MHC class II molecules, thus activating both $CD8^+$ and $CD4^+$ antigen-specific T-cells.[340-342] Third, *Listeria*-derived vaccine vectors may be given orally.[343] In animal models, oral or parenteral immunization with *Lm* engineered to express a number of HIV/SIV antigens induced strong cell-mediated immune responses, but demonstrated little efficacy against a SIVmac239 challenge in macaques vaccinated against Env and Gag in DNA prime-r*Lm* boost regimen.[339,344,345] However, as *Lm* can cause serious infections in neonates, pregnant women and immunocompromised hosts, different attenuation strategies are being undertaken to overcome safety issues associated with the use of live *Lm* as vaccine vector in humans. In particular, a live attenuated *Lm*[346] and a killed but metabolically active *Lm*[347] have been recently developed and shown preserved immunogenicity and efficacy in tumor mouse models.[346-349] In nonhuman primates,

vaccination with an attenuated strain termed *Lmdd* expressing HIV-1 *gag* induced Gag-specific T-cell responses upon oral administration, whereas combined oral/intramuscular administration induced strong Gag-specific systemic and mucosal Ab responses. This difference was also evident for anti-vector Ab, indicating that the route of administration strongly influences the type of response elicited. Of note, a very late boost failed to induce a robust increase of anti-Gag Ab titers, suggesting that anti-vector Ab may severely limit the vaccine immunogenicity.[350] In this regard, an heterologous DNA prime-oral *Lm* boost strategy appears a more promising approach, in that it induced in rhesus macaques mucosal SIV-Gag-specific $CD8^+$ T-cells expressing the $\alpha 4\beta 7$ integrin gut-homing receptor.[351]

Viral Vectors

Safety concerns about live attenuated viruses and inactivated vaccines[352,353] have led scientists to look for better and safer ways of making an AIDS vaccine. Most of the more promising AIDS vaccine candidates currently being developed and tested use viral vectors, viruses that are not harmful and act as the delivery system to carry HIV antigens to the immune system.[354] The potential advantage of the viral vector strategy is to mimic as closely as possible the efficacy of live-attenuated vaccines, while at the same time offering much greater safety. Since several of such vectors are replication competent, the emergence of viral escape mutants may represent a concern. Further, although used as vectors, these are actually viruses that have developed multiple and sophisticated ways to modulate and evade the defense system,[355] which may affect the immunogenicity of the transgene, both qualitatively and quantitatively. Also, some of the proteins of the vector may be highly immunogenic, thus hampering the immunogenicity of the transgene. Thus, a better knowledge of these aspects is critical when designing vaccination approaches based on viral vectors. A number of different viruses have been developed as vectors for vaccines. The different vectors all have their own advantages and disadvantages. Among viral vectors, poxviruses and adenoviruses have received the most attention in the design of HIV vaccine.

Poxvirus Vectors

Several poxviruses, relatives of vaccinia (the smallpox vaccine), are attractive vectors since their large genome allows for the inclusion of multiple heterologous genes, including those encoding antigens, costimulatory molecules and cytokines. Moreover, poxvirus vectors may be used for mucosal immunization.[356] Attenuated vaccinia strains such as MVA and NYVAC (derived from the Copenhagen strain by further deletion of 18 open reading frames encoding molecules implicated in pathogenicity and host-range regulatory functions) are the most frequently used poxvirus vectors. These vectors have been shown to be safe in immunocompromised macaques and in human Phase I/II clinical trials.[357-360] Pre-existing immunity to vaccinia is of a limited concern since its use for smallpox vaccination has ended more than twenty years ago. Most of the recombinant HIV vaccine using poxviruses are effective in nonhuman primate models,[361-364] however, they have much less immunogenicity and less durable immune responses in humans.

Vaccination with rMVA alone has failed to show sufficient immunogenicity in preventive and therapeutic Phase I clinical trials[365,366] and because of the inherent high immunogenicity of the vector MVA is currently used as boost for DNA vaccines (see below). However, therapeutic vaccination with MVA-nef was safe and induced novel immune responses in the majority of the 14 volunteers.[259,367]

Other poxvirus vectors presently being tested include canarypox[368] and fowlpox (for a review see ref. 369). Despite promising results in monkeys.[370] Canarypox vectors expressing different HIV genes have shown limited immunogenicity in humans even at high doses, which were associated to high reactogenicity.[371,372] Results from a Phase II trial recently conducted using the recombinant canarypox ALVAC vCP1452A administered alone or together with rgp120 failed to demonstrate sufficient immunogenicity to grant advancement to Phase III trials.[373] Based on composite data from Phase I and Phase II trials, Aventis and the Thai Ministry of Health, together with the US National Institutes of Health (NIH) and the US Military HIV Research Program, have launched a

Table 6. Preventive and therapeutic ongoing HIV-vaccine clinical trials

Vaccines Based on HIV Structural Gene Products

Vaccine Type	Trial N°	Organizer, Producer	Vaccine Product (Clade)	Phase (I,II,III) Start Date
DNA	HVTN 070	Univ. Pennsylvania	DNA gag, pol, env (B) ± IL-12 or IL-15 DNA	I (preventive) Sep-07
	Env DNA	St. Jude, NIAID	DNA env (A, B, C, D, E)	I (preventive) May-05
Protein	C86P1	SGUL, Richmond Pharmacology, Novartis Vaccines	Prime: HIV-gp140—LT-K63 Boost: HIV-gp140—MF59	I (preventive) Sep-06
	HVRF-380-131004	Moscow Institute Immunology	Env, Gag (B)	I (preventive) Mar-06
Vectored Antigen				
- Poxviruses	HPTN 027	NIAID, Sanofi	ALVAC-HIV vCP1521 env (B, E)	I (preventive) Oct-06
	RV138/VR811	USMHRP	ALVAC- HIV vCP205 env gag, pol (B)	I (preventive) Mar-06
	HIVIS 02	Karolinska Institute, SMI, USMHRP	MVA env (E), gag (A), pol (E)	I (preventive) Jan-06
	RV 158/WR 1143	USMHRP, WRAIR	MVA gp160, gag, pol (A, E)	I (preventive) Jul-05
- Adenoviruses	VRC012	NIAID-VRC	Ad35 env (A) Ad5 env (A)	I (preventive) May-07
	HVTN 054	NIH-VRC	Ad gag, pol (B), env (A, B, C)	I (preventive) Jul-05
Heterologous Prime Boost	HVTN 072	NIAID	Prime: DNA env (A) Boost: Ad5 or Ad35 env (A)	I (preventive) May-07
	HVTN 049	NIAID, Chiron	Prime: DNA/PLG microparticles gag, env (B) Boost: oligomeric V2-deleted gp140 (B)—MF59	I (preventive) Jan-05

continued on next page

Table 6. Continued

Vaccine Type	Trial N°	Organizer, Producer	Vaccine Product (Clade)	Phase (I,II,III) Start Date
Vaccines Based on HIV Structural Gene Products				
	HVTN 042/ANRS VAC019	NIAID, ANRS	Prime: ALVAC-HIV vCP1452 env, gag, pol + CTL epitopes from nef/pol (B) Boost: LIPO-5 or ALVAC (CTL epitopes) from Gag, Pol, Env (B)	I/II (preventive) Apr-04
	RV144	DoD,Thailand MOPH,NIAID TAVEG,Sanofi,VaxGen	Prime: ALVAC env (B, E) Boost: Gag (B), Pol (B), Env (B, E) proteins	III Oct-03
	PO1 AI47490	University of Maryland	Prime: Salmonella Typhi env (B) Boost: Env Protein(B)	I (preventive) Jun-03
	NO1 AI05394	NIAID	Prime: DNA env, gag (A, B, C, E) Boost: Env, Gag protein (A, B, C, E)	I (preventive) May-03
Vaccines based on combined HIV structural and nonstructural gene products				
DNA	N/A	Guangxi CDC	Multiclade DNA plasmids (B, C)	I (preventive) Mar-05
	HIVIS 01	Karolinska Institute, SMI, Vecura	DNA env (A, B, C), gag (A, B), RT (B), rev (B)	I (preventive) Feb-05
	040254; 04-I-0254	NIAID	Multiclade DNA plasmids: gag, pol, nef (B), env (A,B,C)	I (preventive) Aug-04
Protein	108706	GlaxoSmithKline	Gag, Pol, Nef	I/II (preventive) Feb-07
	HVTN 064	DAIDS, Pharmexa-Epimmune	Protein epitopes Env, Gag, Pol, Vpu (B) and/or DNA gag, pol, vpr, nef (A, B, C, D, F, G)	I (preventive) Jan-06

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Table 6. Continued

Vaccines Based on Combined HIV Structural and Nonstructural Gene Products

Vaccine Type	Trial N°	Organizer, Producer	Vaccine Product (Clade)	Phase (I,II,III), Start Date
Vectored Antigen				
- Poxviruses	IAVI C003	ADARC, IAVI, Rockefeller	MVA env/gag-pol, nef-tat (C)	I (preventive) Nov-06
	HIV-POL-001	Bavarian Nordic	MVA HIV polytope vaccine	I (preventive) Oct-06
	IAVI D001	IAVI, Therion	TBC-M4 MVA env, gag, tat, rev,nef ΔRT (C)	I (preventive) Dec-05
	IAVI C002	IAVI-ADARC	MVA env/gag-pol, nef-tat (C)	I (preventive) Jan-05
- Adenovirus (Ad)	AIN504-A5218	NIAID, Merck, HVTN	Ad5 gag, pol, nef (B)	II (therapeutic) Sep-05
	HVTN 057	NIAID	Ad gag, pol (B), env (A, B, C)	I (preventive) Nov-04
- Adeno-associated viruses (AAV)	IAVI A002	IAVI, Targeted Genetics	AAV gag, PR, ΔRT (C)	II (preventive) Nov-05
Heterologous Prime Boost	HIV NAT 064	The National Centre in HIV Epidemiology and Clinical Research, The University of South Wales	Prime: DNA gag, pol, tat/rev, env (A, E) Boost: rFPV gag, pol, tat/rev, env (A, E)	I (preventive) May-07
	HVTN 067	NIAID, Pharmexa-Epimmune	DNA vaccine and MVA (alone or in prime-boost regimen) env, gag, pol, vpu (B); gag, pol, vpr, nef (A, B, C, D, E, G)	I (preventive) Apr-07
	HVTN 069	NIAID	Prime: DNA gag, pol, nef (B), env (A, B, C); Boost: Ad gag, pol (B), env (A, B, C)	I (preventive) Nov-06

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Table 6. Continued

Vaccines Based on Combined HIV Structural and Nonstructural Gene Products

Vaccine Type	Trial N°	Organizer, Producer	Vaccine Product (Clade)	Phase (I,II,III), Start Date
	VRC-011	NIAID, VRC	Prime: DNA gag, pol, nef (B), env (A, B, C) or Ad gag,pol (B),env (A, B, C) Boost: Ad gag, pol (B) env(A, B, C)	I (preventive) May-06
	HVTN 065	NIAID, Geovax	Prime: DNA gag, pro, RT, env, tat, rev, vpu (B); Boost: MVA gag, pol, env (B)	I (preventive) Apr-06
	HVTN 068	DAIDS, NIAID, VRC	Prime: Non replicating Ad gag-pol (B), env (A, B, C) or DNA gag, pol, nef (B), env (A, B, C); Boost: Ad gag-pol (B), env (A, B, C)	I (preventive) Mar-06
	EuroVacc 02	EuroVacc Foundation	Prime: DNA env, gag, pol, nef (C) Boost: NYVAC env, gag, pol, nef (C)	I (preventive) Feb-06
	IAVI V001	IAVI, NIAID	Prime: DNA gag, pol, nef (B), env (A, B, C); Boost: Ad gag, pol (B), env (A, B, C)	I (preventive) Nov-05
	HTVN 063	NIAID, Wyeth	Prime: DNA gag (B) ± IL-15 Boost: DNA gag (B) ± IL-15 or DNA gag (B) ± IL-12	I (preventive) Sep-05
	HTVN 060	NIAID, Wyeth	Prime: DNA gag (B) ± IL-12 Boost: DNA or peptide gag (B) or gag, env, nef (B) + GM-CSF	I (preventive) Aug-05
	HIVIS 03	MUCHS, Karolinska Institute, SMI, Vecura, USMHRP	Prime: DNA env (A, B, C), gag (A, B), RT (B), rev (B); Boost: MVA env (E), gag (A), pol (E)	I/II (preventive) Dec-06

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Table 6. Continued

Vaccines Based on Combined HIV Structural and Nonstructural Gene Products				
Vaccine Type	**Trial N°**	**Organizer, Producer**	**Vaccine Product (Clade)**	**Phase (I,II,III), Start Date**
	RV 172/WR 1218	USMHRP, NIAID	Prime: DNA gag, pol, nef (B), env (A, B, C); Boost: Ad gag, pol (B); env (A, B, C)	I/II (preventive) May-06
	HVTN 204	NIAID, Vical, GenVac	Prime: DNA gag, pol, nef (B), env (A, B, C); Boost: Ad gag, pol (B), env (A, B, C)	II (preventive) Sep-05
	HVTN 055	NIAID, Therion	Prime: MVA env, gag (B) + tat, rev, nef, pol (B); Boost: FPV env, gag (B) + tat, rev, nef, pol (B)	I (preventive) Sep-04
Whole HIV	DCV-02	Hospital Clinic Barcelona	DCs pulsed with inactivated autologous HIV	I/II(therapeutic) Nov-06
	5P01AI57127-2	NIAID	HIV immunogen (whole killed gp120-depleted HIV inactivated)	I/II (therapeutic) Oct-05
	2000456-01H	Ottawa Health Research Institute	Remune (vaccine) and ALVAC (vaccine)	I/II (therapeutic) Sep-05

ADARC: Aaron Diamond AIDS Research Center; **ANRS**: Agence Nationale de Recherches sur le SIDA (France); **DAIDS:** Division of AIDS; **DoD:** US Department of Defense; **EuroVacc:** European Vaccine Effort Against HIV/AIDS; **Guangxi CDC:** Guangxi Centre for Disease Control and Prevention**; HPTN:** HIV Prevention Trials Network; **HVTN:** HIV Vaccine Trials Network; **IAVI:** International AIDS Vaccine Initiative; **MoPH:** Ministry of Public Health; **MUCHS:** Muhimbill University College of Health Science; **NIAID**: US National Institute Allergy and Infectious Diseases; **NIH**: US National Institutes of Health; **SGUL:** St George's, University of London; **SMI**: Swedish Institute for Infectious Disease Control; **St. Jude**: St. Jude Children's Research Hospital; **TAVEG**: Thai AIDS Vaccine Evaluation Group; **USMHRP**: US Military HIV Research Program; **VRC**: Vaccine Research Center; **WRAIR**: Walter Reed Army Institute of Research; **MVA**: Modified Vaccinia Ankara; **FPV;** fowlpoxvirus; **vCP:** viral Canarypox; **LT-K63**: nontoxic mutant of *Escherichia coli* heat labile enterotoxin (LT); **PLG:** Polylactide- coglycolide; **GM-CFS**: Granulocyte-macrophage colony stimulating factor.

Phase III trial with a HIV-1 Env-based vaccine in which a canarypox prime is followed by boosting with the VaxGen gp120 protein (Table 6).

Similarly, despite promising results in the monkey model,[374] a Phase I therapeutic trial with a fowlpox vector expressing Gag and Pol failed to display any immunogenicity of the transgenes, although it induced anti-vector Abs.[375] Immunogenicity was demonstrated in a more complex therapeutic trial in which immunization with fowlpox expressing several HIV antigens was combined with HIV lipopeptides (synthetic fragments of HIV proteins associated with lipids that facilitate the induction of a cellular immune response) and followed by administration of IL-2, confirming the intrinsic weak immunogenicity of this vector.[376] Fowlpox vectored vaccines performed poorly also in heterologous DNA prime-fowlpox boost approaches both in monkeys and Phase I preventive or therapeutic trials.[375,377,378] Subsequent studies in the monkey model suggest that the poor immunogenicity observed in human as compared to monkeys might be due to the vaccine dose used, which might be insufficient to trigger adequate responses.[379] However, higher production costs, multiple inoculations and reactogenicity are serious obstacles to scaling up vaccine dosing and might hamper further development of vaccines of this type.

Adeno and Adeno-Associated Vectors

Adenoviral vectors have a broad host range and infect both proliferating and quiescent cells. The tropism of adenoviruses for mucosal epithelium makes them extremely attractive as vectors for HIV vaccine development since they can been delivered orally or intranasally and induce mucosal immune responses.[380] Recombinant adenovirus vectors can accommodate larger inserts, mediate transient but high levels of protein expression and can be easily produced at high titers. Furthermore, adenoviruses targets DCs in which they up-regulate costimulatory molecules and MHC class II expression and induce production of Th-1 and pro-inflammatory cytokines.[381,382] Of note, Adenovirus-based vaccine candidates have produced the most impressive cellular immune responses seen so far.[383] Both replication-competent and—incompetent vectors are being developed as vaccine against HIV. However, while replication-competent adenovirus vectors induce stronger and more persistent humoral and cellular immune responses compared to the nonreplicating vectors, there are safety concerns about their use in clinical trials.[383-385]

The replication-incompetent recombinant adenovirus Type 5 (rAd5) is a modified form of Ad5, the virus that causes some forms of the common cold. It is replication-defective to enhance safety and represents one of the most promising viral vectors for HIV vaccines. However, prior exposure to Ad5 may boost anti Ad-5 antibody response blunting the expression of the transgene and the percentage of volunteers responding to the vaccine. This anti-vector immunity may represent a major problem in the developing world, where the prevalence of prior exposure to Ad5 is greatest.[386] This has prompted the development by Merck, Crucell and Transgene, in collaboration with IAVI, of candidate vaccines based on less prevalent human Adenovirus serotypes (Ad6, Ad35, Ad11, or Ad 24) to replace the Ad5 vector in fusion HIV trials.[387-390] Another approach to circumvent pre-existing immunity to Ad5 has been to modify the vector by substituting key neutralizing epitopes on the surface of viral capsid proteins with those from the less prevalent serotype Ad48. Such chimeric vector is called Ad5HVR48.[391]

Finally, pre-existing immunity to adenovirus may be overcome by heterologous prime-boost strategies, including DNA priming followed by adenovirus vector boosting,[96,392] or the use of different adenovirus serotypes, including the above mentioned Ad5HVR48, for priming and boosting.[393,394]

Upon extensive testing in nonhuman primates Merck found out that intramuscular vaccination with SIV Gag delivered by Ad5 (E1- deleted) was superior to DNA or MVA at inducing CTL responses and at protecting against disease following pathogenic intravenous challenged with SHIV-89.6P,[141,142] but not against an intrarectal challenge with SIVmac239.[143] Noteworthy, the limited protection upon SIVmac239 occurred in the presence of T-cell responses that correlated with protection in the former study.[144] Co-immunization with Ad5 carrying Gag and Env was less effective than Gag alone at controlling infection in rhesus macaques challenged intravenously with

SHIV-89.6P[238] confirming the detrimental role of CTL responses to Env observed in the natural infection.[63] In order to increase the breadth of response against HIV-1 and to improve the vaccine efficacy, replication defective Ad5 vectors carrying pol and nef were constructed and a Phase IIb trial started in 2004 in which 3,000 high risk individuals were immunized intramuscularly 3 times with replication defective Ad5 vectors expressing gag, pol and nef (Ad5MRKAd5 trivalent). This trivalent vaccine was generally safe and well tolerated at all doses studied and immunogenic eliciting responses against the 3 antigens included in the vaccine. However, preliminary data indicate that vaccination did not protect from infection or lowered viral loads. Further, there was an apparent higher susceptibility to infection in vaccinees with pre-existing immunity to the vector.[146,395] Reduced expression and immunogenicity of the transgenes, as suggested by the much lower proportion of vaccinees responding to all the three HIV antigens (Gag, Pol and Nef) in the group with pre-existing immunity to Ad5 as compared to vaccinees with no pre-existing immunity, immune activation generated by the response to the vector and/or an increase of HIV-specific target T-cells induced by the vaccine[148] are some of the hypotheses that have been proposed to explain these findings. Thus, although very preliminary, overall preclinical and clinical data may suggest that SIV is a more rigorous challenge virus and a better predictor of vaccine efficacy in human and that boosting of pre-existing immunity to the vector may actually enhance the susceptibility to infection as compared to placebo or vaccinees with no pre-existing immunity. Another nonreplicative adenoviral vector (deleted of the genes coding for E1 and E3 proteins) has been developed by NIH Vaccine Research Center (VRC), together with a DNA-based vaccine. These are multicomponent vaccines, which express the Env glycoprotein from clades A, B and C and the Gag, Pol and Nef proteins from clade B and are designed for use in a DNA prime-Ad5 boost regimen strategy.[396] Despite differences in the vaccine design, initiation of Phase II trials has been postponed to late 2008, when a better understanding of the reasons of the Merck's vaccine failure will clarify whether vaccination with the VRC candidate would pose the same risks.

Replication-competent adenoviral vectors have also been developed as vehicles for AIDS vaccines.[397] Studies in both chimpanzee and rhesus macaque models have demonstrated that priming with replicating Ad recombinants encoding HIV or SIV genes followed by boosting with viral protein subunits elicits potent humoral, cellular and mucosal immune responses.[385,398-405] Of note, vaccination of Rhesus macaques with Ad vectors expressing HIV-1 Tat and Env conferred a strong protection against a challenge with the pathogenic SHIV 89.6P, which was superior to that provided by a larger vaccine formulation including SIV Gag and Nef in addition to HIV-1 Tat and Env.[268] This underscores the importance of properly selecting the antigens to combine together and provides one of the strongest evidence in favor of the Tat + Env vaccine.

Other viral vectors used as AIDS vaccines in clinical trials include Adeno-associated viruses (AAV) which are not adenoviruses but are often found in adenovirus infections.[406,407] These vectors are currently used in Phase I and Phase II clinical trials.[9] However, the weak immunogenicity recorded in a multicentric Phase I study[408] has led to halting the initiation of Phase II trials in India and spurred a debate on the ethics of conducting the ongoing Phase II trials in Africa in the face of such disappointing Phase I results. The slightly better immunogenicity recorded at the highest dose tested in the Phase I trials may suggest that a dose increase could solve this problem. However, recent data indicate that, in mice, vaccination with high doses of AAV expressing Gag induced Gag-specific effector $CD8^+$ CTLs that were weak producers of IFN-γ, expressed exhaustion markers and failed to become memory cells. Transition to the memory phenotype and restoration of full functionality was achieved upon adoptive transfer, suggesting that chronic exposure to the trangene might have been the cause of the CTL dysfunction.[409]

Other Viral Vectors

Other viral vectors have been tested as vaccine vectors for HIV-1 and have shown various degrees of success (for a review see ref. 410). Among them, the alphaviruses include weakened forms of three viruses named Venezuelan Equine Encephalitis (VEE), Sindbis (SIN) and Semliki Forest Virus (SFV). The first alphavirus vector candidate, AlphaVax's VEE, is designed as a replicon

particles containing self-replicating RNA encoding the VEE replicase proteins and expressing a gene of interest in place of the viral structural protein genes. An appealing feature of alphaviruses is their known ability to induce apoptosis of transduced cells, favouring DC cross-priming.[411] These replicon particles have shown protection against other viruses and have elicited significant cell-mediated and antibody immune responses with SIV antigens, perhaps due to the propensity of the vector to target antigen-presenting cells.[412] The VEE vector is currently being tested in clinical trials.[413]

Viruses belonging to the rhabdovirus family and in particular the vesicular stomatitis virus (VSV) are also being used. These vectors offer the advantage to be highly flexible, easy to manipulate and able to express large and multiple foreign genes.[414] Intramuscular vaccination of mice with a single-cycle vector expressing HIV Env elicited strong Env-specific humoral and cellular responses.[415] Furthermore, immunization of macaques with recombinant VSVs (rVSVs) expressing SIV Gag and HIV Env has been reported to protect from pathogenic SHIV89.6P.[416,417] These promising results have led to the development of rVSV for use in humans.[418] However, since the prototypic rVSV vector was found to be insufficiently attenuated for clinical evaluation, novel highly attenuated vectors have been designed, which are less neurovirulent and more immunogenic than the prototypic rVSV vector.[419]

Other potentially powerful vaccine delivery systems are represented by Polioviruses.[420] Both replication-competent and replication-deficient recombinants have been shown to be immunogenic in nonhuman primates when used through various routes of immunization, including mucosal delivery.[421] However, restrictions to the use of these vectors include the stability and size of heterologous gene inserts[422] and the presence of high levels of pre-existing immunity to polio vectors in the general population.

Replication-competent and replication-defective herpesviruses (HSVs), including HSV-1, represent suitable vaccine vectors against AIDS. Important advantages include broad host cell range, high infectivity and easy of production of high-titer stocks of viruses, long-term expression of foreign antigens and stimulation of both humoral and cellular arms of the immune system. Vaccination with replication-competent or replication-defective HSVs vaccine vectors expressing SIV Env and Nef, protected macaques against a challenge with SIVmac239.[423] However, the overall toxicity and the pre-existing immunity against the vector may represent a safety issue for their use in humans and current strategies focus on the development of replication-incompetent viruses used in prime-boost regimen with DNA.[424]

New Particulate Delivery Systems

Microparticles have been effectively used for many years as particulate delivery systems for drugs, therapeutic proteins and various types of vaccines including recombinant proteins, plasmid DNA, peptides and other vaccine components (e.g., immune potentiators).[425,426] Among antigen-loaded microspheres, injectable, biodegradable polymeric particles prepared with poly(d,l-lactide-co-glycolide) (PLG) or poly(d,l-lactide) (PL) polymers represent a successful method for in vivo delivery of peptide, protein or DNA antigens.[427] Both particles have been shown to be effective, especially for oral delivery.[428] Antigen instability and manufacturing difficulties have been overcome by the recent findings that adsorption rather than microencapsulation of the antigen onto PLGA is easier, cheaper and ensures better antigen stability.[429] In comparison to standard aluminum-based adjuvants, these microspheres have many desirable features, including the ability to enhance the bioavalaibility of the antigen, allowing pulsating antigen release and to reduce the number of doses in the immunization schedule, mimicking the conventional prime-boost regimen. Furthermore, for adjuvanting vaccines against intracellular pathogens and cancer, selective targeting of PLGA microparticles to DCs has been achieved and induction of CTLs has been attained in both small animals and nonhuman primates.[430] In particular PLGs have been demonstrated to enhance the immunogenicity of DNA vaccines to HIV-Gag and HIV-Env in rhesus macaques.[431] PLG particles are currently being evaluated in a gag + env DNA/PLG prime-ΔV2 Env protein boost preventive Phase I trial.[189]

Two novel classes of biocompatible core-shell anionic microspheres have been used as an efficient delivery system for vaccination with the Tat protein.[432] These microspheres, synthesized by dispersion polymerization, are characterized by an increased shelf-life and the capability of reversibly adsorbing native proteins at their surface. In particular, these microparticles consist of negatively charged microspheres, made of either poly(styrene) or poly(methyl methacrylate) and in which hemisuccinated poly(vinyl alcohol) or Eudragit L100/55 were used, respectively, as steric stabilizers.[432] These microspheres prevented Tat from oxidation, maintaining the native and biologically active conformation required for vaccine efficacy and efficiently delivered Tat intracellularly. In the mouse model, delivery of Tat by these microspheres was safe and immunogenic.[433,434]

VLPs

Virus-like particles (VLPs) are self-assembling, nonreplicating, nonpathogenic particles that are similar in size and conformation to intact virions.[435,436] VLPs offer a number of advantages over conventional protein immunogens and have been therefore considered as an ideal HIV vaccine candidate.[437] In fact, these particles can be easily produced in large amount in heterologous expression systems (baculovirus, vaccinia virus) and easily purified. In addition, since VLPs lack regulatory proteins as well as infectious genetic material, they are both replication- and infection-incompetent, making VLPs safer than live-attenuated viruses. Further, VLPs express viral proteins in their native conformation and generally induce more effective humoral and cellular immune response than their soluble counterparts, in both the systemic and mucosal immune compartments.[437-439]

However, due to their nonreplicating properties, VLPs are less effective at inducing cellular immune responses as compared to live-attenuated viruses or replicating viral vector vaccines. For this reason, novel approaches are being developed in order to increase their immunogenicity, including DC targeting.[440] The mucosal administration of VLP vaccines has also emerged as a promising strategy to elicit mucosal and systemic anti-HIV humoral and cellular immune responses.[441]

To date, numerous types of VLPs have been produced utilising the ability of capsid and envelope proteins to self-assemble into highly organised particulate structures. In particular, the Gag protein is required for their assembly, budding and release from host cell. VLPs, based on HIV-1 p55gag, presenting the entire gp120 molecule from an Ugandan clade A HIV-1 isolate, have been shown to induce strong systemic and mucosal humoral and cellular immune responses in mice.[442,443] More recently, IN administration in a mouse model of these VLPs together with the Eurocine L3 mucosal adjuvant (a monoglycerides/fatty acid lipid suspensions)[444] in a heterologous (DNA + VLPs) prime-boost strategy induced higher titers of NAbs and stronger anti-Env T-cell responses as compared to vaccination with adjuvanted VLPs only.[445] Further, a combined multiepitope VLP-based HIV vaccine (Combi HIVvac) carrying both B- and T-cell epitopes (from HIV-1 Env, Gag, Pol and Nef proteins) resulted safe and highly immunogenic in mice.[446] Of interest, vaccination of rhesus macaques with p55gag VLPs in the absence of adjuvant induced broad, durable anti-Gag CTLs.[447] However, therapeutic vaccination with HIV-1 p17/p24:Ty virus-like particles, which contain part of the HIV-1_{IIIB} Gag sequence and are produced by expressing a *TYA:p17/p24* fusion gene in yeast[448] did not appear to slower HIV-1 disease progression,[449] or to impact $CD4^+$ T-cell decline in patients with advanced HIV infection.[450]

Prime Boost Strategies

Many of the vaccine studies combine various approaches in a prime-boost fashion to optimize the immune responses elicited. A heterologous prime boost strategy is the administration of one type of vaccine (the primer is usually DNA) followed by the administration of another form of the vaccine (the booster is usually recombinant proteins or attenuated viral vectors). The goal of this approach is to complement the priming by a different stimulation of the immune system to enhance the body's overall immune response to HIV, a result that may not be achieved with a single type of vaccine. For example, while DNA or microparticles are optimal for inducing T-cell responses, they are poor inducers of Ab, which, however, are readily induced upon boosting with protein or a recombinant vector. Another advantage of this strategy is that it circumvents the

relatively common and detrimental immunodominance of the vector that may result in a reduced immunogenicity of the transgene and the impossibility to use the same vector twice because immunity to the vector strongly reduces or prevents the transgene expression. To overcome this latter problem sequential immunizations with different viral vectors have been used as an alternative prime-boost approach, as reported in nonhuman primate models against SHIV[451] and SIV[143,144] challenges. DNA prime-viral vector boost approaches may also be exploited to target mucosal site either because of the intrinsic tropism of the vector or because they can be applied mucosally. A variety of protocols using alternative viral vectors for both priming and boosting have also been reported, both alone and in combination with DNA and have been successful at limiting disease progression, but not at offering protection against infection. For example, DNA priming followed by a recombinant MVA expressing multiple HIV proteins did not prevent but effectively controlled infection upon challenge with pathogenic SHIV89.6P in rhesus macaques.[361,452] Based on the promising results in monkeys, GEOVAX is currently testing in 4 different Phase I trials a DNA prime-MVA boost approach in which priming with DNA encoding Tat, Rev, Vpu and Gag is followed by boosting with MVA expressing Env, Gag, Protease and RT. Preliminary data indicate good safety and CTL responses in over 50% of the vaccinees[453] and a Phase II trial is planned for 2008. Similarly, McMichael and coworkers at the Oxford University have shown in Phase I studies that DNA prime-MVA boost HIV vaccines are well-tolerated and immunogenic, but the percentage of volunteers responding to the vector and the durability of $CD8^+$ cell-mediated responses have not matched so far the responses observed with the rAd5 vector.[132,366,454] However, the lack of solid correlates of protection and the large body of evidence showing that natural control of infection is not necessarily associated with strong immune responses should not impede advancement of these type of vaccines to Phase II trials.

International Networking to Ease and Accelerate HIV/AIDS Vaccine Development

The first Phase I trial of an HIV vaccine was conducted in the USA in 1987. Since then, more than 50 candidate vaccines have been tested in about 100 Phase I/II clinical trials, involving more than 35,000 healthy human volunteers. Two Phase III trials have been completed and a third one is in progress. The vast majority of these vaccine candidates, including those tested in Phase III trials, were based on structural HIV-1 proteins and primarily aimed at inducing NAbs. Most of the efforts to develop and evaluate HIV vaccines is borne by the NIH, CDC and WRAIR in the USA and by ANRS in France, with strong help from the International AIDS Vaccine Initiative (IAVI) in New York (http://www.iavi.org), the European Union (EU), initiatives in WHO (http://www.who.int/en) and UNAIDS (http://www.unaids.it) and the recent commitment of the Bill and Melinda Gates Foundation for a Global Enterprise (http://www.gatesfoundation.org/GlobalHealth/Pri_Diseases/HIVAIDS).

The HIV Vaccine Trial Network (HVTN) established by NIAID in 2000, with 25 clinical sites in four continents, represents a major resource for clinical HIV vaccine research (http://www3.niaid.nih.gov/about/organization/daids). The EU has also established a comprehensive program aimed at strengthening integration of science among countries of the EU and promoting, among the others, vaccine development against poverty diseases (i.e., HIV/AIDS, TB, Malaria). The AIDS Vaccine Integrated Project (AVIP) (http://www.avip-eu.org), Mucosal Vaccines for Poverty Related Diseases (MUVAPRED) (http://www.mucosalimmunity.org/muvapred/index.asp) and the European Vaccine effort against HIV/AIDS (EUROVAC) (http://www.eurovac.net), are among the most important projects recently cofunded by the EU. In addition, the European and Developing Countries Clinical Trials Partnership (EDCTP) has been created with the aim of helping developing countries to build up their capacity in testing the efficacy of new drugs, microbicides and vaccines.

Conclusion

Several advancements have been made over the past few years to improve vaccine strategies aimed at inducing protection against HIV. Ideally, the aim of an effective vaccine would be to produce sterilizing immunity in all recipients. However, also a vaccine able to control rather than prevent the infection might have important benefits, reducing HIV levels in the body, delaying progression to AIDS and initiation of anti-retroviral therapy and reducing the chance of HIV transmission.

The current knowledge suggests that an effective HIV candidate should induce both humoral and cellular immune responses, to ensure durable immunological memory and to boost both the adaptative and innate immune system. The latter one is particularly important at mucosal sites of HIV transmission.[455] One of the major impediments to the development of an HIV-1 vaccine is the lack of knowledge of the immune correlates of protection. Although studies of MEU and LTNPs continue to provide valuable information on mechanisms of natural protection, which can then be applied to vaccine design, it should be kept in mind that immune responses in LTNPs may represent a correlate of preservation of immune competence in a host containing infection rather than the actual factors controlling the virus. Natural resistance to infection has been attributed to a combination of genetic, innate and acquired immune system-mediated mechanisms.[20] Therefore, a novel approach for treatment and/or prevention of HIV infection might be represented by the manipulation of these restriction factors in order to improve and broaden their activities.[456] The early containment of HIV-1 and SIV replication in acutely infected individuals and monkeys is temporally associated with the emergence of a virus-specific CTL response and high levels of circulating CTLs are associated with good clinical status in chronically infected individuals[457,458] and acutely infected monkeys.[459] Importantly, experimental in vivo depletion of $CD8^+$ T-cells in monkeys, abrogated control of SIV replication during primary infection and the animals died after a rapidly progressive disease course.[460-462] While this has generally been interpreted as the definitive proof of the key role of $CD8^+$ T-cells in the containment of infection, it should be reminded that also NK cells express the CD8 molecule and their experimental depletion may contribute to the loss of virus control.

Interestingly, loss of control of infection has been reported also upon B-cell depletion during primary SIVmac infection of rhesus monkeys,[460,463,464] suggesting that either Abs are indeed crucial for containing the virus even at the very beginning of the infection or, more in general, that severe disturbance of a component of the immune system disrupts the proper function of system as a whole, underscoring the integrated nature of the defense system[360,463,465-469] and the contribution of multiple arms to an effective control of infection (for a review see ref. 470).

Because of safety concerns, traditional immunization approaches, including those based on live attenuated and inactivated viruses, have been almost abandoned. Vaccine candidates based on purified or synthetic proteins are mainly developed to induce NAbs, whereas recent advances in molecular biology and genetic engineering have led to the development of a new generation of vaccines, which includes DNA- and microorganism-vectored vaccines, which are primarily aimed at inducing T-cell responses. In this regard, vaccinia viruses, canarypox constructs, replication-competent and replication-defective adenovirus vectors are the main live vectors currently being evaluated. The success of these vectors is believed to depend also on their capability to trigger innate immune responses, which would induce proper adaptive immunity. Although replication-competent adenoviruses have the advantage of persistently infecting the host and stimulating the immune system,[383] safety issues need to be fully addressed before their advancement to clinical trials. The recent failure of the Merck trial clearly indicates that even replication-defective adenoviral vectors may be harmful in the presence of pre-existing immunity to the vector. Thus, DNA vaccines with increased immunogenicity and microbial vectors that circumvent pre-existing immunity to the vector are needed. In this regard, optimization and further exploration of new adjuvants for DNA and protein antigens are currently being heavily pursued.[471] VLPs have also been employed as multi-epitope vaccine since they offer the advantages of (i) mimicking the virion without having the safety concerns of live-attenuated viruses, (ii) inducing both mucosal and systemic immune

responses, (iii) activating both endogenous and exogenous antigen presentation pathways (MHC class I and II, respectively) and (iv) maintaining the antigens in their native conformation.

Effective vaccination may ultimately require two or more vaccines used in conjunction (heterologous prime-boost strategies), an approach to vaccine development that differs from traditional vaccine design and is presently the preferred strategy for many vaccine candidates against HIV/AIDS (Table 6). In this regard, there is a general agreement that when exploited combined, the vaccine components used for the prime and the boost are expected to stimulate a broader and more diversified immune response than using any of them repeatedly. In addition, the single use of a viral or bacterial vector will avoid the interference, on a second administration, of pre-existing immunity to the vector.

Effective anti-HIV/AIDS vaccines may require targeting of several HIV-1 antigens. Among these multi-component vaccines novel minimalistic vaccination strategies, combining structural (ΔV2-Env) and nonstructural (Tat or Nef) proteins have been rationally designed to induce NAbs and T-cell responses against key early and late HIV antigens. In particular, preclinical testing of the Tat/ΔV2-Env combination in macaques has shown efficacy (ref. 268 and Ensoli B, in preparation) and clinical trials with this vaccine candidate will start in 2008.

However, HIV vaccine development still faces significant challenges. The availability of an effective HIV vaccine requires scientific and public-health efforts and the establishment of Consortia such as the "European Consortia for HIV vaccine development" (including AVIP, MUVAPRED, VIAV), the "Neutralizing Antibody Consortium"; the "HIV Global Enterprise", an international Consortium of nongovernamental and governamental organizations.[472] Clinical trials must also be performed with appropriate ethical rules, especially in developing countries, avoiding duplication of efforts, using standardized genetic inserts as immunogens and implementing immunological assays for preclinical and clinical testing to compare candidate vaccines. This is important because the laboratory assays used to assess immune responses may not be comparable, severely hampering decisions about which candidates to pursue for further testing. In addition, new knowledge about the immune response to HIV is raising concerns that current assays overlook important aspects of those immune responses.

Open questions remain to be answered, such as how to induce high titers of NAbs; whether any of the vaccines being currently developed will elicit cellular immune responses that will correlate with protection from infection or disease progression; the type (poly- or mono-functional) of $CD4^+$ and $CD8^+$ T-cell responses elicited by the vaccines currently being developed; the magnitude, breath and durability of the vaccine-induced $CD4^+$ and $CD8^+$ T-cell responses; the best combination of vaccines that in the prime-boost immunization strategies will stimulate an immune response similar to that thought to confer protection from disease progression.

The challenges the scientific community still faces are formidable. However, looking back, enormous progresses have been made in each aspect of vaccine development, from basic science to clinical testing, that let us be optimistic about the eventual generation of effective preventive and therapeutic vaccines against HIV/AIDS. While vaccines able to slow disease progression and decrease transmission rate should be at reach in the medium term, recent advancements in the generation of Env-based immunogens, their association to key regulatory or accessory HIV-1 proteins and present reconsideration of the several Ab effector functions, make us hoping that even a sterilizing vaccine may be not too far distant.

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