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Nonstructural HIV proteins as targets for prophylactic or therapeutic vaccines

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By the end of 2004, more than 20 HIV-1 vaccine candidates will have entered clinical testing in at least 30 trials worldwide. Almost half of these vaccines include nonstructural HIV-1 gene products. This represents an important innovation in the HIV vaccine field, because until 9 years ago not even preclinical testing in small animal models had been carried out with such immunogens. This review briefly discusses the experimental evidence that provides the rationale for the use of nonstructural HIV-1 gene products as vaccine antigens, and summarizes the current status and the future development of these novel vaccines.

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Abbreviations

Ab	antibody
AIDS	acquired immunodeficiency syndrome
AVIP	AIDS Vaccine Integrated Project
CTL	cytotoxic T lymphocyte
DC	dendritic cell
Env	envelope
HAART	highly active antiretroviral therapy
HIV	human immunodeficiency virus
MHC	major histocompatibility complex
MVA	modified vaccinia Ankara
nAb	neutralizing antibody
SHIV	simian–human immunodeficiency virus
SIV	simian immunodeficiency virus

Introduction

The human immunodeficiency virus (HIV) epidemic has caused over 20 million deaths worldwide and currently affects about 40 million people (UNAIDS, 2004; http://www.unaids.org) with a dramatic socioeconomic impact, particularly on developing countries (UNAIDS, 2004). To date, the sole effective weapon against HIV and acquired immunodeficiency syndrome (AIDS) is therapy. However, highly active antiretroviral therapy (HAART) is not accessible worldwide and the most in need (i.e. the infected population of poor countries) often have no treatment. In addition, toxic effects and the appearance of resistant viral variants, particularly in the absence of a correct education for full compliance, can render HAART difficult to manage (reviewed in [1]). The urge for an effective prophylactic and/or therapeutic HIV vaccine is therefore soaring.

Early vaccine strategies based upon inactivated or attenuated virus have been abandoned as ineffective or unsafe, respectively (reviewed in [2] and [3]). The focus then shifted towards the development of viral subunit vaccines based on HIV structural proteins. In particular, the HIV envelope (Env) proteins (gp41 and gp120) contain most of the HIV neutralization-sensitive epitopes and would, in principle, represent the ideal components of a neutralizing antibody (nAb)-based candidate vaccine capable of inducing sterilizing immunity. Unfortunately, to date, Env-based vaccines have failed because of the complex structure of Env, its high variability, and the difficulty of generating broadly reactive high-titer nAbs (reviewed in [4]).

After several unsuccessful attempts to induce humoral responses conferring sterilizing immunity (reviewed in [4]) and the appreciation, after the introduction of HAART, that progression to disease can be halted, new approaches have been undertaken with the goal of inducing cellular immunity capable of containing, even though not preventing, the infection and of blocking disease development. Vaccines based on various combinations of the structural proteins Env, Gag and Pol were partially protective (reviewed in [4]) in preclinical studies, yet never provided sterilizing immunity. Moreover, the emergence of cytotoxic T lymphocyte (CTL) viral escape mutants voided the benefits of vaccination (reviewed in [5]). By contrast, pre- or post-virus exposure immunotherapy with nAbs provided sterilizing immunity against simian-human immunodeficiency virus (SHIV) challenge in nonhuman primates (reviewed in [6]), suggesting that potent nAb responses can be protective.

It is now believed that both cellular and humoral immunity are necessary for controlling and, possibly, eradicating infection at an early stage or for preventing disease progression. With this aim, the efficacy of single or multicomponent vaccines including several virus subunits has been investigated in preclinical and clinical trials. Several of these novel AIDS vaccine strategies employ

nonstructural (regulatory or accessory) HIV or simian immunodeficiency virus (SIV) proteins and will be reviewed here.

Nonstructural HIV proteins as AIDS vaccine targets — the rationale

In addition to six structural proteins — group antigens (Gag), polymerase (Pol), Env, protease (Pro), reverse transcriptase (RT) and integrase (Int) — the HIV genome encodes the small regulatory proteins Tat and Rev and four accessory proteins, Nef, Vif, Vpr and Vpu. Tat and Rev are early products and are essential for productive infection and virus replication. Tat is a transcriptional transactivator of HIV gene expression and is crucial for viral replication, cell-to-cell virus transmission and pathogenesis (reviewed in [7••]); Tat is required for the expression of structural proteins and for the production of infectious virus particles (reviewed in [7••]).

Infected cells release Tat very early after infection by a leaderless secretory pathway (reviewed in [7••]). This extracellular Tat can enter both infected and uninfected cells, where it promotes HIV replication or modulates the expression of cellular genes (transcription factors, cytokines and genes that regulate the cell cycle and that are important for HIV replication), respectively (reviewed in [8]). Extracellular Tat also binds to specific cell-surface receptors and phosphorylates tyrosine kinases, thus activating specific signal transduction pathways, and upregulates the expression of chemokine receptors and the HIV co-receptors CCR5 and CXCR4 (reviewed in [7••] and [8]). Extracellular Tat also has important effects on immunoregulatory functions (reviewed in [7••]). In particular, bioactive soluble Tat can selectively bind and enter both immature and mature dendritic cells (DCs), drive immature DC maturation and activation towards a T-helper 1 (Th-1)-inducing phenotype [9••], gain access to the major histocompatibility complex (MHC) class I pathway of presentation [10,11], and modulate the proteasome catalytic subunit composition, modifying the hierarchy of the CTL epitopes presented in favor of subdominant and cryptic epitopes [12••].

Both cellular and humoral Tat-specific immunity can 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 [13]. 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 [14]. Of utmost importance, these data have been recently confirmed in patients enrolled before seroconversion (i.e. shortly after exposure to the virus and before anti-HIV antibodies [Abs] became detectable in the serum) and in which a strong temporal correlation between anti-Tat CTL appearance (as early as 8 days post-infection), viral load decline, and CD4 T cell recovery was found [15••]. Furthermore, anti-Tat Abs can sequester the extracellular protein, thus preventing the extracellular Tat-driven enhancement of infection and immune dysregulation associated with them.

Consistent with this hypothesis, the presence of Tat-specific CTL responses correlates with non-progression to AIDS both in SIV-infected monkeys and in HIV-positive individuals [16••,17–19]. Furthermore, 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 non-progression to AIDS (reviewed in [7••]; G Rezza *et al.*, personal communication). Whether this indicates that neutralization of extracellular Tat can 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 and likely to induce broad immune responses, as suggested by the recent observation that sera from Ugandan and South African individuals infected with non-clade B HIV-1 strains cross-react with the Tat protein of an HIV-1 clade B strain [20••].

Rev is also absolutely required for HIV replication. In fact, it induces the transition from the early to the late phase of gene expression and proviruses that lack Rev do not produce virions. However, spontaneously occurring mutations in Rev, which reduce but do not abolish HIV late (i.e. structural) gene expression to levels undetectable by CTLs, have been reported and might represent a possible mechanism to escape immune recognition [21•]. Like Tat, Rev is often targeted by CTLs in HIV-positive individuals [22] and is broadly conserved among different HIV-1 clades in its functionally constrained and immunodominant domains at the N terminus [23•], thus representing a potentially valuable vaccine target.

The accessory protein Nef is also expressed early during the virus replication cycle, is essential for high-titer virus production and is involved in HIV pathogenesis [24,25]. Nef downregulates the CD4 and MHC class I molecules [26], thus diminishing the host immune response and control of HIV infection. In macrophages, Nef induces the production of several soluble factors responsible for the recruitment and activation of resting T lymphocytes and increased virus replication [27,28]. In DCs, Nef promotes the expression of DC-SIGN (DC-specific intercellular adhesion molecule 3 grabbing nonintegrin), a transmembrane molecule present on the cell-surface and capable of binding HIV particles and delivering them to T lymphocytes. Therefore, the Nef-enhanced expression of DC-SIGN on DC membrane favors retention of the infectious virus and cell-to-cell transmission [29•]. Consequently, immune containment of Nef functions

could significantly contribute to controlling HIV infection. The other accessory proteins, Vpr, Vpu and Vif, which are expressed late in the virus life cycle, are also immunogenic [30–33] and have been included in multicomponent candidate vaccine formulations to broaden virus-specific immunity [31–33].

Vaccines based on single nonstructural HIV proteins

Early expression, a critical role in the virus life cycle, the conservation of immunogenic epitopes in different HIV-1 subtypes, and immunomodulatory functions are the key features shared by Tat, Nef and Rev that make them attractive vaccine targets.

Tat-based vaccines

Tat has been recently proposed as a novel vaccine target on the basis of its early expression and immune-modulating properties. Systemic or mucosal immunization strategies, based on biologically active native Tat or inactivated Tat, respectively, have been evaluated in animal models and humans, with the aim of blocking viral infection at a very early stage (abortive infection) or to contain viremia and prevent progression to AIDS.

Vaccines based on biologically active Tat

The native, biologically active form of Tat or its DNA has been proven to be safe and immunogenic in preclinical and clinical studies (Table 1 and references therein). Of note, the administration of wild-type Tat protein or DNA to SHIV-infected monkeys with AIDS (B Ensoli *et al.*, unpublished) or HIV-infected individuals [34,35] did not fuel viral replication or cause any further reduction in $CD4^+$ T-cell counts, ruling out the most important safety concern.

In a two-arm study [36,37], most cynomolgus macaques immunized with the Tat protein or *tat* DNA were protected against intravenous challenge with the pathogenic SHIV89.6P that encodes Tat of HIV-1 (Table 2). All controls became overtly infected, with high viral load and steep $CD4^+$ T-cell decline. In 9 out of 12 vaccinated animals neither plasma viremia nor $CD4^+$ T-cell count decline were detected, even though low numbers of proviral DNA copies were sporadically found in a few animals [36,37]. Protection correlated with the presence of Tat-specific CTLs and tumor necrosis factor α production by $CD8^+$ T cells. Both Tat protein and *tat* DNA elicited long-term specific memory T cells in protected monkeys, which did not show signs of systemic infection throughout a 104-week follow-up or even upon two boostings with tetanus toxoid, a stimulus known to activate T cells and to increase virus replication [38•]. Thus, vaccination of nonhuman primates with biologically active Tat alone was safe, induced humoral and cellular immune responses, and protected against intravenous challenge with a pathogenic SHIV. Phase I clinical trials with the biologically active Tat protein are ongoing in Italy to evaluate the safety and immunogenicity of preventive or therapeutic vaccination in HIV-negative or HIV-positive individuals, respectively. In view of future phase II/III trials, virologic, immunologic and feasibility studies are also ongoing in Africa.

The development of second-generation Tat-based vaccines is aimed at obtaining easily administrable formulations with increased shelf-life and efficacy (Table 1). Novel approaches based on new delivery systems [39,40•, 41–43] or employing codon-optimized or ubiquitin-tagged *tat* DNA [44] triggered potent cellular responses against Tat in mice (Table 1). As HIV is mainly transmitted via the mucosal route, strategies to obtain mucosal vaccines to elicit protective topical responses have also been undertaken (Table 1). Intranasal vaccination with biologically active Tat protein alone was also safe and immunogenic in mice [45•,46•,47].

Vaccines based on inactivated Tat or Tat peptides

Although immunization with native Tat is safe (reviewed in [7••]) (Tables 1, 2 and 3), mutants lacking the transactivating activity or a chemically inactivated Tat protein (Tat toxoid) were also evaluated as candidate vaccines (Tables 2 and 3). Transdominant negative mutants of *tat* were as immunogenic as wild-type *tat* DNA in mice [48] and are being evaluated in nonhuman primate trials. Tat toxoid was also safe and immunogenic in rodents, macaques and HIV-negative or HIV-positive individuals [49–55] (Tables 1, 2 and 3). In efficacy studies in rhesus macaques (Table 2), vaccination with Tat toxoid (or native Tat) was partially protective against intrarectal challenge with the pathogenic SHIV89.6PD [52]. Animals that controlled infection mounted both humoral and cellular responses before challenge, suggesting the involvement of both arms of immunity in containing infection. In another study [55], immunization with Tat toxoid or biologically active Tat did not protect against intravenous challenge with SHIV89.6P, despite the presence of virus-specific humoral and cellular immunity (Table 2). Differences in vaccine design and formulation, adjuvant, schedule and route of immunization, and dose of challenge virus may account for these discrepancies (Table 2).

Vaccination with Tat peptides has also been evaluated and found safe and immunogenic in several preclinical trials (Tables 1 and 2). Candidate vaccine formulations based on a multipeptide conjugate, including three functional domains of Tat from HIV-1 groups M and O [56], or on several HIV-1 clade B Tat peptides [57] elicited broadly reactive Abs capable of recognizing the whole protein and blocking its biological functions. In another immunization study, vaccination of monkeys with Tat peptides encompassing Tat B-cell epitopes has provided evidence for a role of anti-Tat Abs in containing plasma viremia [58].

Table 1

Safety and immunogenicity of vaccines based on nonstructural HIV/SIV antigens in small animal models.

Model[c]	Immunization[a]				Immune responses[b]			Refs
	Immunogen	Dose (No. of vaccine administrations)[d]	Adjuvant[e]	Route[f]	Ab[g]	Cellular		
						Th[h]	CTL[i]	
Mouse	HIV *nef* DNA	2 μg (1)	None	im	ND	ND	+	[59]
Mouse	HIV *tat*, *rev* or *nef* DNA	1–100 μg (1–3)	None	im, ie	+	+	ND	[84]
Mouse	HIV *nef* DNA or HIV *nef, rev, env* and *gag* ± *tat* DNA	2–100 μg (1–3)	None	im, ie	+	+	ND	[84]
Hu-PBL-SCID mouse	HIV *rev* and *env* DNA or *nef*, *rev* or *tat* DNA	1–100 μg (1–3)	None	ip, id	+	+/−	ND	[84]
Mouse	HIV Tat, Rev or Nef protein	5–50 μg (2)	CFA, RIBI	sc	+	ND	ND	[84]
Mouse	HIV *tat, rev, nef*, *env* and *gag* DNA	5–10 μg (3)	None	im, id, ie, in, ito	+	+	ND	[85]
Mouse	HIV *tat, rev, nef*, *env* and *gag* DNA	20 μg (2–3)	None	in	+	+	ND	[86]
Mouse	HIV Tat toxoid	5–20 mg (2)	CFA, IFA	im	+	ND	ND	[51]
Mouse	HIV *tat* DNA (inactivated mutants)	100 μg (6)	None	im	+/−	+	ND	[48]
Mouse	HIV *vif, vpu* and *nef* DNA	100 μg (2–3)	None	im	ND	+	+	[33]
Mouse	HIV Tat multiple peptide conjugate	10–100 μg (3)	CFA, IFA	ip	+	+/−	ND	[56]
Mouse	HIV Tat toxoid	25–100 μg (3–9)	IMS, CHI, PLG	im, in, o	+	ND	ND	[54]
Mouse	HIV *tat*, *rev* or *nef* DNA in pBN-vector	1–25 μg (6)	None	ie, im, id	+/−[j]	+	+	[87]
A2.01 transgenic mouse	HIV *tat*, *rev*, *nef* DNA alone or combined	10–50 μg (2)	None	im	+	+	ND	[66]
Mouse	HIV Tat protein	10 μg (4)	LT-R192G	in	ND	ND	+	[47]
Mouse	DNA, SFV and MVA encoding SIV *gag* and *env* and HIV (clade A) *env, gag, pol* and *nef* CTL epitopes (HIVA)	50 μg DNA (1–2) ± 5×10^8 pfu MVA (1) or 50 μg DNA (1–2) ± 10^6 IU SFV (1) or 5×10^8 pfu MVA (1–2) or 10^6 IU SFV (1–2) or 10^6 IU SFV (1) + 5×10^8 pfu MVA (1)	None	im, id	ND	ND	+	[88]
Mouse	HIV Tat protein	10 μg (4)	LT-R72, Alum, IFA	in, sc	+	+	+	[46•]
Mouse	HIV Tat protein	10 μg (4)	None, MALP-2	in, id	+	+	+	[45•]
Mouse	HIV Tat protein	0.2–10 μg (4)	CFA, RBCs	ip	+	+	+	[89•]
Mouse	HIV *tat* DNA	1–30 μg (6)	Cationic block copolymers	im	−	+	+	[40•]
HLA transgenic mouse	DNA encoding HIV *gag, pol, env, rev, nef* and *vpr* epitopes	100 μg (2)	None	im	ND	ND	+	[90]
Mouse	HIV Tat protein	10 μg (3)	Alum	sc	+	+	ND	[42]
Mouse	HIV Tat protein	5 μg (2)	ANPs, Alum, Lipid A	sc	+	+	+	[42]
Mouse	DNA and MVA encoding consensus HIV (clade A), *env*, *tat, rt* and *nef* sequences + SIV Tat SL8 and *Plasmodium berghei* mouse CTL epitopes (RENTA)	25 μg DNA (1) or 10^6 pfu MVA (1) or 25 μg DNA (1) + 10^6 pfu MVA (1)	None	im	ND	ND	+	[76]
Mouse	DNA and MVA encoding SIV *gag* and *env* and HIV (clade A) *env, gag, pol* and *nef* CTL epitopes (HIVA) + DNA and MVA encoding consensus HIV (clade A) *env*, *tat, rt* and *nef* sequences + SIV Tat SL8 and *P. berghei* mouse CTL epitopes (RENTA)	25 μg DNA (HIVA or RENTA) (1) or 5×10^4 pfu MVA (HIVA or RENTA) (1) or 25 μg of each DNA (1) + 5×10^4 pfu of each MVA (1)	None	im	ND	ND	+	[76]
Mouse	HIV *tat* DNA (codon-optimized; ubiquitin-tagged and codon-optimized)	100 μg (4)	None	im	ND	+	+	[44]

Table 1 Continued

Model[c]	Immunization[a]				Immune responses[b]			Refs
	Immunogen	Dose (No. of vaccine administrations)[d]	Adjuvant[e]	Route[f]	Ab[g]	Cellular		
						Th[h]	CTL[i]	
Mouse	HIV Tat protein	0.5–2 μg (3)	None, AMPs, Alum	im	+	+	+	[k]
Rabbit	HIV Tat toxoid	1–250 mg (2)	CFA, IFA	id, im	+	ND	ND	[51]
Rabbit	HIV Tat protein	100 μg (4)	CFA, IFA	id	+	ND	ND	[91]

[a]No toxic effects were observed in the studies cited in the table. MVA, modified vaccinia Ankara; SFV, Semiliki Forest virus. [b]+, ≥ 50% responders; +/−, <50% responders; −, no responders; ND, not done. [c]Hu-PBL-SCID mice are mice reconstituted with human peripheral blood lymphocytes. [d]IU, infectious units; pfu, plaque forming units. [e]CFA, complete Freund's adjuvant; RIBI, an oily adjuvant emulsion containing bacterial and mycobacterial cell walls; IFA, incomplete Freund's adjuvant; IMS, trade name of an oily adjuvant emulsion; CHI, nanoparticles of chitosan on which the Tat toxoid was loaded; PLG, polylactide-*co*-glycolide microparticles on which the Tat toxoid was loaded; LT-R72 and LT-R1926, non-toxic mutants of *Escherichia coli* heat labile enterotoxin; Alum, aluminium hydroxide or phosphate; AMPs, anionic microparticles; ANPs, anionic nanoparticles; MALP-2, 2 kDa macrophage-activating lipopeptide from *Mycoplasma fermentans*; RBCs, red blood cells. [f]im, intramuscular; ie, intraepidermal, by gene gun delivery; ip, intraperitoneal; id, intradermal; sc, subcutaneous; in, intranasal; ito, intratongue; o, oral. [g]Antibody (IgG, IgA) responses measured by enzyme-linked immunosorbent assay (ELISA). [h]T-helper (Th) response evaluated by lymphoproliferative assay or intracellular cytokine staining. [i]CTLs were evaluated either by cytotoxic assay or interferon-γ secretion/expression (ELISA, enzyme-linked immunospot or intracellular cytokine staining). [j]Ab response detected only for Nef. [k]A Caputo, unpublished.

Nef-based vaccines

Vaccination with HIV *nef* DNA was first reported to induce specific CTLs in mice by Asakura *et al*. [59] (Table 1). More recently, vaccination with SIV *nef* DNA was shown to be safe and to protect macaques against SIV-induced disease [60] (Table 2). The safety and immunogenicity of HIV-1 Nef delivered as plasmid DNA or as recombinant viral vector (modified vaccinia Ankara, MVA) as a therapeutic vaccine was also proven in HIV-infected individuals [34,61] (Table 3). Strong Nef-specific CD4 T-cell responses were elicited that were comparable with those generally detected in long-term non-progressors, suggesting that therapeutic vaccination with Nef might provide valuable HIV-specific immune responses [61].

Rev-based vaccines

So far, Rev alone as vaccine antigen has been used only for therapeutic vaccination. Administration of *rev* plasmid DNA to HIV-1-infected individuals was safe and immunogenic [34], in both HAART-treated and HAART-naïve individuals [35] (Table 3).

Vaccines based on multiple nonstructural HIV proteins

Multiantigen vaccination based on nonstructural HIV proteins aims at inducing broad immunity to multiple viral targets, to maximize the control of infection while minimizing the emergence of escape viral mutants. Tat-Rev combined vaccines protected macaques against systemic infection upon intravenous or intrarectal challenge with SIVmac32H or SHIV-BX08, respectively [62,63] (Table 2). Comparative studies in an SIV monkey model [64•] showed that vaccination with Tat and Rev elicits more effective CTL responses than immunization against structural Gag and Pol proteins (Table 2), underscoring the importance of immune-targeting early virus proteins.

In a monkey pilot study, Vpr suppressed the benefits of *nef* DNA vaccination by compromising antigen-specific T-cell immunity [60,65] (Table 2). In fact, co-immunization with SIV *nef* and *vpr/vpx* (SIV protein with Vpr-like functions) abolished the protective effects of vaccination with *nef* alone against intrarectal challenge with pathogenic SHIV [60] (Table 2). By contrast, immunization with *tat*, *rev* and/or *nef* DNA elicited humoral and cellular responses in mice [66] (Table 1). In macaques, vaccination with Retanef, a polyprotein comprising Rev, Tat and Nef, induced cellular responses to all gene products [67,68] (Table 2). In asymptomatic HIV-1-infected individuals intramuscular administration of plasmid DNA encoding Rev, Tat and Nef was safe and improved cellular (T-helper and CTL), but not humoral, immune responses [69•] (Table 3).

Vaccines based on multiple structural and nonstructural HIV proteins

Additional promising approaches to AIDS vaccines are based on the exploitation of antiviral immunity to both structural and nonstructural (late and early) HIV products. Antibody and cellular responses generated through a DNA prime-protein boost regimen (which consists of the consecutive administration of the same immunogen both as DNA and protein and is aimed at enhancing both specific humoral and cellular responses) including Tat, Gag and Env protected macaques against infection and/or SHIV89.6P-induced disease [70•]. Also, a vaccine combining Env with a Tat–Nef fusion protein, but not vaccines based on the single component, was effective at containing virus replication in macaques challenged with SHIV89.6P [71•]. Similar vaccine approaches based

Table 2

Safety, immunogenicity and efficacy of vaccines based on nonstructural HIV/SIV antigens in non-human primate models.

	Immunization[a]				Pre-challenge immune responses[b]			Challenge			Protection from infection[h]	Protection from disease[i]	Refs
	Immunogen	Dose (No. of vaccine administrations)	Adjuvant[c]	Route[d]	Ab[e]	Cellular		Virus	Dose MID_{50}	Route[d]			
						Th[f]	CTL[g]						
Monkey	HIV Tat, Rev, Nef, Env and Gag, DNA and protein	10 μg DNA (4) + 50 μg of protein (2) for each antigen	Gold particles, RIBI	ie, im	+	+	–[j]	SHIV-4	10 (MID_{100})	iv	No	N/A	[92]
Monkey	HIV Tat protein	6–10 μg (8) + 16 μg in ISCOMs (1)	RIBI, Alum, ISCOMs	sc, id	+	+	+	SHIV89.6P	10	iv	Yes (5/7)	Yes (5/7)	[36]
Monkey	SFV and MVA viral vectors expressing SIV Tat and Rev	$10^{8–9}$ $TCID_{50}$ SFV-*tat* and *rev* (2) + $10^{8–9}$ $TCID_{50}$ MVA-*tat* and *rev* (2)	None	im	ND	ND	ND	SIVmac32H	50	iv	Yes (2/2)	NA	[62]
Monkey	DNA and MVA encoding SIV *gag* and *env* and HIV *env, gag, pol* and *nef* CTL epitopes	8 μg DNA (2) + 5×10^8 pfu MVA (2)	None	id	ND	ND	+[k]	SIVmac251	20	ir	Yes (1/3)	Yes (1/3)	[74]
Monkey	HIV Tat protein	10–40 μg (5)	Poly-phosphazene, IFA	id, im	+	+	ND	SHIV89.6P	2500 ($TCID_{50}$)	ir	Yes (2/4)	Yes (1/4)	[52]
Monkey	HIV Tat toxoid ± Env (vaccinia virus and protein)	Tat toxoid 20–80 μg (5), gp120 100 μg (2)	Poly- phosphazene, IFA	id, im	+	+	ND	SHIV89.6P	2500 ($TCID_{50}$)	ir	Yes(6/12)	Yes(2/12)	[52]
Monkey	HIV Tat peptides (2)	0.1–0.5 mg of each peptide (3–4)	CFA, IFA	im	+	ND	ND	$SHIV_{33}$ or $SHIV_{33A}$	50/200	iv	No	N/A or No	[58]
Monkey	HIV Tat protein	20 μg (3)	Alum, MPL	sc	+	+	+	SHIV89.6P	≤100	iv	No	No	[l]
Monkey	HIV *tat* DNA and Tat protein	0.2–1 mg DNA (8) + 16 μg protein in ISCOMs (1)	ISCOMs	id, im	+	+	+	SHIV89.6P	10	iv	Yes (4/5)	Yes (4/5)	[37]
Monkey	MVA encoding SIV *env*, *gag*, *pol*, *tat*, *rev*, *nef*	1×10^8 pfu MVA (3)	None	im	+[m]	–	–	SIVsm	10	ir	No	N/A	[79]
Monkey	MVA and SFV encoding SIV *env*, *gag*, *pol*, *tat*, *rev*, *nef*	1×10^8 pfu SFV (2) + 1×10^8 pfu MVA (2)	None	im	+[m]	–	+	SIVsm	10	ir	No	N/A	[79]
Monkey	HIV *env*, *rev* and SIV *gag*, *pol* DNA	0.2–0.5 mg of each DNA (5 + 2 + 2)	None	im	ND	+/–	+/–	SHIVIIIB + SIVmac239 + SHIV89.6P	10+10 + 10	iv	Yes (3/8) Yes (3/3) Yes (3/3)	Yes (3/8) Yes (3/3) Yes (3/3)	[93]
Monkey	DNA and NYVAC viral vector expressing SIV Rev, Tat and Nef chimeric gene *(Retanef)*	4 mg DNA (3) + 10^8 pfu NYVAC (1)	None	id, im	ND	ND	+	N/A	N/A	N/A	N/A	N/A	[67,68]
SIVmac251-infected monkey	DNA or NYVAC viral vector expressing SIV Rev, Tat and Nef chimeric gene *(Retanef)*	4 mg DNA (1) or 10^8 pfu NYVAC (1)	None	id, im	ND	ND	+	N/A	N/A	N/A	N/A	N/A	[67,68]
Monkey	Ad5hr-SIV*env/rev*	5×10^8 pfu (2)	None	in + o, it	ND	ND	+	N/A	N/A	N/A	N/A	N/A	[94•]
Monkey	Ad5hr-SIV*env/rev* + SIV Env booster	5×10^8 pfu Ad5hr (2) + 100 μg gp120 (2)	MPL-SE	in, o + im	ND	ND	+	N/A	N/A	N/A	N/A	N/A	[94•]
Monkey	[p]Ad5hr-SIV*env/rev* and [p]Ad5hr-SIV*nef* + SIV Env booster	5×10^8 pfu of each Ad5hr (2) + 100 μg gp120 (2)	MPL-SE	in, o + im	ND	ND	+	N/A	N/A	N/A	N/A	N/A	[94•]
Monkey	DNA and MVA encoding SIV *gag* and *env* and HIV (clade A) *env gag, pol* and *nef* CTL epitopes (HIVA)	8 μg DNA (2) + 5×10^8 pfu MVA (2)	None	id	ND	ND	+	N/A	N/A	N/A	N/A	N/A	[95]

Monkey	SIV *gag, pol, vif, vpx* and *vpr* and HIV *env, tat* and *rev* DNA (+ MVA-*gag, pol, nef* booster)	0.25/2.5 mg (2) DNA (+ 2×10^8 pfu MVA) (1)	None	id, im	ND	ND	+[n]	SHIV89.6P	NA	ir	No	Yes (24/24)	[31,32]
Monkey	HIV Tat protein	100 μg (4)	IFA	im	+	+	+	SHIV89.6P	30	iv	No	Yes (1/8)	[53,55]
Monkey	HIV Tat toxoid	100 μg (4)	IFA	im	+	+	+	SHIV89.6P	30	iv	No	Yes (1/8)	[53,55]
Monkey	SIV *tat* DNA and MVA	32 μg DNA (3) + 5×10^8 pfu MVA (1)	Gold particles (for DNA)	ie, id, ir	ND	+	+	SIVmac239	3160 ($TCID_{50}$)	ir	No	No	[96]
Monkey	SFV and MVA vectors expressing SIV Tat and Rev	10^{8-9} $TCID_{50}$ SFV-*tat* and *rev* (2) + 10^{8-9} $TCID_{50}$ MVA-*tat* and *rev* (2)	None	im	+	+	+	SIVmac32H	50	iv	Yes (2/4)[o]	NA	[64•]
Monkey	SFV and MVA vectors expressing HIV Tat	10^8 $TCID_{50}$ SFV-*tat* (2) + 10^8 $TCID_{50}$ MVA-*tat* (2)	None	sc, im	–	ND	–	SHIV-BX08	10	ir	Yes (1/4)	N/A	[63]
Monkey	SFV and MVA vectors expressing HIV Tat and Rev	10^8 $TCID_{50}$ SFV-*tat* and *rev* (2) + 10^8 $TCID_{50}$ MVA-*tat* and *rev* (2)	None	sc, im	–	ND	+	SHIV-BX08	10	ir	Yes (1/4)	N/A	[63]
Monkey	HIV *tat* and *rev* DNA and MVA vectors expressing HIV Tat and Rev	0.1 mg (5) + 0.25 mg (2) DNA + 10^8 $TCID_{50}$ MVA-*tat* and *rev* (2)	IL-12 DNA (500 μg)	id, im	–	ND	–	SHIV-BX08	10	ir	Yes (2/4)	N/A	[63]
Monkey	SIV *nef* DNA	0.1–0.5 mg of each plasmid (9)	None	im	ND	ND	ND	SIVmac239	NA	ir	No	Yes (2/2)	[60]
Monkey	SIV *nef*, *vpr* and *vpx* DNA	0.1–0.5 mg of each plasmid (9)	None	im	ND	ND	ND	SIVmac239	NA	ir	No	No (2/2)	[60]
Monkey	HIV Nef-Tat fusion protein and SIV Nef protein	20 μg (3)	AS02A	im	+	ND	ND	SHIV89.6P	5–50	iv	No	No	[71•]
Monkey	HIV Nef-Tat fusion protein, SIV Nef and Env proteins	20 μg (3), Env 100 μg (3)	AS02A or AS06	im	+	ND	ND	SHIV89.6P	5–50	iv	Yes (5/8)	Yes (5/8)	[71•]
Monkey	SIV *tat, rev, nef* and *gag* DNA and MVA vectors expressing SIV Tat, Rev, Nef and Gag	32–64 μg of each DNA (6), 10^8 IU of each vector	Gold particles (for DNA)	ie, o, id, in, iv	ND	+	+	SIVmac239	10	ir	No	NA	[72]
Monkey	HIV Tat, DNA and protein	0.5 mg DNA (3) + 25 μg protein in ISCOMs (3)	ISCOMs	im	+	+	+	SHIV89.6P	50	iv	No	No	[70•]
Monkey	HIV Tat and Env and SIV Gag, DNA and protein	0.5 mg DNA of each Ag (3) + 25 μg protein in ISCOMs (3)	ISCOMs	im	+	+	+	SHIV89.6P	50	iv	Yes (1/4)	Yes (4/4)	[70•]
Monkey	DNA and MVA encoding SIV *gag* and *env* and HIV (clade A) *env, gag, pol* and *nef* CTL epitopes (HIVA) + DNA and MVA encoding HIV (clade A) *env*, *tat, rt* and *nef* sequences + SIV Tat mouse CTL epitopes (RENTA)	1 mg of each DNA (HIVA or RENTA) (2) + 5×10^7 pfu of each MVA (2)	None	im	ND	ND	+	N/A	N/A	N/A	N/A	N/A	[76]
Monkey	SIV *env* and *rev* DNA or [p]Ad5hr-SIV *env/rev* + [p]Ad5hr-SIV *env/rev* booster and SIV Env	3 mg DNA (1 or 2) and/or 10^9 pfu Ad5hr (1) + 10^9 pfu Ad5hr (1) + 100 μg gp120 (2)	None	in, it, id	+[q]	+[q]	+[q]	N/A	N/A	N/A	N/A	N/A	[97]
Monkey	DNA, SFV and MVA encoding SIV *gag*, *pol*, *tat*, *rev*, *nef* and *env*	100 μg DNA/each Ag (1) + 10^8 IU SFV/each Ag (2) + 10^8 $TCID_{50}$ MVA of each Ag (1)	None	id, sc, im	+	+	+	SIVmac251	50	ir	Yes (3/4)	NA	[78••]
Monkey	DNA and MVA or vaccinia virus expressing HIV Tat, Rev, Nef, Gag, Env and RT	0.25–0.5 mg DNA/each Ag (2–3) + 10^8 pfu MVA (2) or vaccinia (1)	GM-CSF	im	+	ND	+	SHIV-4	25	iv	Yes (2/4)	N/A	[77]

Table 2 Continued

	Immunization[a]				Pre-challenge immune responses[b]			Challenge			Protection from infection[h]	Protection from disease[i]	Refs
	Immunogen	Dose (No. of vaccine administrations)	Adjuvant[c]	Route[d]	Ab[e]	Cellular		Virus	Dose MID_{50}	Route[d]			
						Th[f]	CTL[g]						
Monkey	DNA and MVA or vaccinia virus expressing HIV Tat, Rev, Nef, Gag, Env and RT	0.25–0.5 mg DNA/each Ag (2–3) + 10^8 pfu MVA (2) or vaccinia (1)	GM-CSF	im, ir, o	+	ND	+	SHIV-4	25	iv	Yes (4/4)	N/A	[77]
Monkey	HIV *tat* DNA and Tat protein	1 mg DNA (7) + 25 μg protein in ISCOMs (2)	None	im	+	+	+	N/A	N/A	N/A	N/A	N/A	[r]
Monkey	HIV Tat protein	6 μg (11)	None	id	+	+	+	N/A	N/A	N/A	N/A	N/A	[r]

[a]No toxic effects were observed in the studies cited in the table. MVA, modified vaccinia Ankara; NYVAC, New York vaccinia; SFV, Semiliki Forest virus. [b]+, ≥ 50% responders; +/−, <50% responders; −, no responders; ND, not done. [c]RIBI, an oily adjuvant emulsion containing bacterial and mycobacterial cell walls; Alum, aluminium hydroxide or phosphate; ISCOMs, immunostimulating complex composed of *Quillaja saponins,* cholesterol, phospholipids and protein; IFA, incomplete Freund's adjuvant; CFA, complete Freund's adjuvant; MPL, monophosphoryl lipid A; MPL-SE, squalene-based stable emulsion containing MPL; IL-12, interleukin-12; AS020A, an oil-in-water emulsion containing 3D-MPL and the saponin QS21; AS06, aluminium hydroxide plus CPG sequence-containing oligonucleotide 1826; GM-CSF, granulocyte-monocyte colony-stimulating factor. [d]ie, intraepidermal by gene gun delivery; im, intramuscular; sc, subcutaneous; id, intradermal; in, intranasal; o, oral; it, intratracheal; ir, intrarectal; iv, intravenous. [e]Ab, antibody (IgG, IgA) responses measured by ELISA. [f]T-helper (Th) response evaluated by lymphoproliferative assay. [g]CTLs were evaluated either by cytotoxic assay, interferon-γ secretion/expression (ELISA, ELIspot or intracellular cytokine staining) or tetramer staining. [h]Protection from infection is defined as undetectable or transiently detectable low acute viremia. [i]Protection from disease is defined as a significantly lower peak viremia and/or viral setpoint and maintenance of normal CD4 T cell counts as compared to infected control animals. Fractions refer to the number of animals protected from infection or disease per total number of treated animals in the group. NA, not available; N/A, not applicable. [j]CTLs anti-Tat, Rev or Gag were not evaluated. [k]Only CTLs to the SIV Gag epitope p11C were measured. [l]JW Shiver, report at the 18th annual symposium on non-human primate models for AIDS, 2000. [m]Ab response evaluated only for Env, Gag and Nef. [n]Only anti-Gag responses were evaluated. [o]2/4 animals with undetectable plasma viral load but transiently positive cellular viremia. [p]Ad5hr, adenovirus type 5 host range. [q]Immune responses were evaluated only against Env. [r]B Ensoli, unpublished.

Table 3

Phase I/II clinical trials evaluating vaccines based on nonstructural HIV/SIV antigens.

Recipients	Immunization[a]				Immune response[b]			Refs
	Immunogen	Dose (No. of vaccine administrations)[c]	Adjuvant[d]	Route[e]	Ab[f]	Cellular		
						Th[g]	CTL[h]	
HIV-positive or HIV-negative individuals	HIV Tat toxoid	50–100 mg	IFA	im	+	+/−	ND	[49]
HIV-positive individuals	HIV *tat*, *rev* or *nef* DNA	100 μg (3)	None	im	+/−	+	+	[34,35]
HIV-negative individuals	*env/rev* DNA	0.1–1 mg (4)	None	im	–	+/−	+/−[i]	[98]
HIV-positive individuals	MVA expressing HIV Nef	5×10^8 IU MVA (3)	None	sc	+/−	+	+/−	[61]
HIV-positive individuals	HIV *tat*, *rev* and *nef* DNA	100 μg (1) + 300 μg (1) + 600 μg (1) for each antigen	None	im	−	+	+/−	[69•]
HIV-negative individuals	DNA and MVA encoding SIV *gag* and *env* and HIV (clade A) *env, gag, pol* and *nef* CTL epitopes (HIVA)	100 or 500 μg DNA (2) or 5×10^7 pfu MVA (2) or 100 or 500 μg DNA (2) + 5×10^7 pfu MVA (1)	None	im, id	−	ND	+	[75]

[a]No toxic effects were observed in the studies cited in the table. MVA, modified vaccinia Ankara. [b]+, ≥ 50% responders; +/−, <50% responders; –, no responders; ND, not done. [c]IU, infectious units; pfu, plaque-forming units; [d]IFA, incomplete Freund's adjuvant; [e]im, intramuscular; sc, subcutaneous; id, intradermal; [f]Ab, antibody (IgG, IgA) responses measured by ELISA; [g]T-helper (Th) response evaluated by lymphoproliferative assay or intracellular cytokine staining; [h]CTLs were evaluated either by cytotoxic assay or interferon-γ secretion/expression (ELISA, enzyme-linked immunospot or intracellular cytokine staining). [i]CTL responses were mostly CD4-T-cell mediated.

on various combinations of nonstructural (Tat, Rev and Nef) and structural (Gag and Env) HIV-1 gene products are being evaluated in preclinical and clinical trials in Europe within the AIDS Vaccine Integrated Project (AVIP; http://www.iss.it). The aim of AVIP is to exploit both the antigenicity and adjuvanticity (i.e. immunomodulatory activities) of Tat and to elicit effective immunity to both early and late HIV products.

In chronically infected macaques under antiretroviral therapy (ART), immunization with SIV Retanef, Gag and Pol expanded virus-specific $CD4^+$ and $CD8^+$ T-cell responses to both regulatory and structural proteins and correlated with the containment of viremia upon ART discontinuation [67,68] (Table 1). A DNA/MVA prime-boost regimen [72] based on structural (Gag) and nonstructural (Tat, Rev and Nef) proteins was only partially effective against SIV mucosal challenge, containing acute viremia until week three post-exposure. Although pre- and post-challenge cellular responses were detected against all vaccine viral antigens, nAbs against the challenge virus were barely detectable six months after virus exposure, suggesting that multispecific CTLs can contain acute viremia but cannot control chronic infection in the absence of nAbs [72] (Table 2). A phase I clinical trial based on HIV-1 clade B Tat, Rev, Nef and Gag is planned (http://chi.ucsf.edu/vaccines). In another study, combined vaccination with Tat, Nef and gp120 protected macaques from heterologous SHIV-induced disease, in contrast to immunization against Tat and Nef or gp120 alone (Table 2) [71•], and is under clinical evaluation (http://chi.ucsf.edu/vaccines).

Co-immunization against SIV Gag, Pol, Vif, Vpx and Vpr, and against HIV-1 Env, Tat and Rev contained viremia levels and protected all vaccinated rhesus macaques from acute disease upon intrarectal challenge with the pathogenic SHIV89.6P, whereas all controls developed AIDS [31,32]. Phase I clinical trials based on vaccination with DNA encoding HIV-1 clade B structural *env*, *gag*, *pro* and *rt*, and nonstructural *vpu*, *tat* and *rev* are ongoing at Emory University or planned by GeoVax in the United States (http://chi.ucsf.edu/vaccines).

Vaccination with multiple conserved CTL epitopes from structural and nonstructural HIV antigens has been recently developed with the aim of inducing CTLs capable of preventing or containing HIV infection at very early stages. A DNA prime-MVA boosting vaccination with a string of partially overlapping epitopes from Env, Gag, Pol and Nef has been shown to be safe and immunogenic in mice [73] and to induce anti-Gag CTLs, preventing infection upon intrarectal challenge in one of three macaques [74]. More recently, a clade A HIV poliepitope vaccine (HIVA) consisting of 73% of the Gag protein fused to a string of 25 partially overlapping CTL epitopes has been generated for phase III efficacy trials in Kenya. Results from phase I studies indicate that HIVA is safe and immunogenic in humans [75]. Very recently a novel fusion protein from consensus HIV clade A sequences of Tat, RT, Nef and gp41 (RENTA) has been engineered and found to be safe and immunogenic in mice and monkeys, alone or combined with the HIVA multiepitope [76]. A DNA prime-MVA boosting approach combining RENTA and HIVA is planned to enter clinical trials.

Conclusions

Overall, nonstructural vaccine targets have proven to be safe and immunogenic in preclinical and clinical trials. Promising efficacy data have also been obtained in non-human primates, where immunity to nonstructural viral proteins provided or contributed to long-lasting protection against challenge with pathogenic SIV or SHIV strains [36,37,52,62,63,64•,70•,71•,74,77,78••]. In particular, novel vaccine strategies combining nonstructural and structural antigens aim at inducing broad cellular and humoral immunity to eliminate infected cells and to neutralize infectious virions or the activity of infection-enhancing viral proteins, respectively. How many and which of the HIV-1 proteins have to be combined to obtain a broadly effective HIV/AIDS vaccine remains to be determined. The optimization of formulations and immunization schedules will also be important, especially in view of the risk that structural, immunodominant viral products such as Gag and Env might diminish the generation or maintainance of responses against small regulatory proteins. This potential complication was suggested by preclinical studies that included Gag, Env, Tat, Rev and Nef [70•,77,78••,79] and by the pattern of immune responses detected in the course of the natural infection [22,80•–82•]. Such difficulties may be overcome by a rational vaccine design, where priming with less immunogenic antigens precedes immunization with stronger immunogens or vaccination is performed simultaneously with all antigens but at different sites. These strategies are currently under evaluation within AVIP, an EU Commission awarded integrated project (6th Framework Programme). In this project, several combinations of nonstructural and structural gene products are being tested in preclinical studies to select the best candidates that will enter phase I preventive and therapeutic trials. Preliminary data in monkeys indicate that sequential immunization with Tat protein followed by Gag elicits humoral and cellular responses to both antigens (B Ensoli, unpublished). The polyvalent vaccine approach is, to date, the most promising for both therapeutic and prophylactic efficacy, as indicated by preclinical studies (Table 2). Although several phase I/II clinical trials have already proven the safety and immunogenicity of multi-component vaccines based on early and late viral genes, phase IIb and III trials are urgently needed for validation of the proof-of-concepts (e.g. the identification of immune correlates of protection, the determination of immune response durability, the validation of nonhuman primates as animal models for AIDS vaccines, and evaluation of the impact of HIV variability on cross recognition) and to demonstrate efficacy. The recent call for the constitution of a 'global HIV vaccine enterprise' [83] represents a major advance and it could provide the HIV vaccine field with the critical mass necessary to speed up vaccine development and testing, hopefully answering the many questions that still challenge us.

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