Problems and emerging approaches in HIV/AIDS vaccine development

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According to recent estimates, 39.5 million people have been infected with HIV and 2.9 million have already died. The effect of HIV infection on individuals and communities is socially and economically devastating. Although antiretroviral drugs have had a dramatically beneficial impact on HIV-infected individuals who have access to treatment, it has had a negligible impact on the global epidemic. Therefore, the need for an efficacious HIV/AIDS vaccine remains the highest priority of the world HIV/AIDS agenda. The generation of a vaccine against HIV/AIDS has turned out to be extremely challenging, as indicated by > 20 years of unsuccessful attempts. This review discusses the major challenges in the field and key experimental evidence providing a rationale for the use of non-structural HIV proteins, such as Rev, Tat and Nef, either in the native form or expressed by viral vectors such as a replicating adeno-vector. These non-structural proteins alone or in combination with modified structural HIV-1 Env proteins represent a novel strategy for both preventative and therapeutic HIV/AIDS vaccine development.

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1. Background

Early after the discovery of HIV as the cause of AIDS, researchers predicted that a preventative vaccine was just around the corner. It became clear that the task was very difficult to accomplish and, despite almost 20 years of efforts, the search for an effective HIV vaccine still continues. The wrong prediction was most likely dictated by the protective efficacy against challenge with pathogenic simian type D retrovirus (SRV) obtained in rhesus monkeys following immunisation with the whole inactivated virus or the SRV Env glycoprotein. It was shown that the protection correlated with the establishment of binding and cross-neutralising antibodies to different SRV serotypes [1]. At that time, however, very little was known about the complexity of HIV infection and the importance of HIV accessory (*nef*) and regulatory (*rev*, *tat*) genes in the pathogenesis of AIDS. Rev, Tat and Nef are essential for productive infection, virus replication and propagation. In addition, these proteins also cause immune dysfunction and contribute importantly to AIDS pathogenesis. Recent advancements in the understanding of the role of structural and non-structural viral proteins in causing HIV immunopathogenesis have had important consequences on HIV vaccine design leading to the development of novel vaccine candidates based on or including non-structural viral proteins.

Table 1. Selected host and virus factors in HIV infection.

	Mechanisms that control infection	Refs
Host factors		
HLA and allelic variation	Certain alleles and allele variants are associated with different susceptibility to HIV infection or rates of disease progression	[170-172]
Chemokine receptors	Individual carrying the alleles for CCR5Δ32 are less susceptible to HIV infection and are more likely to become LTNPs	[173]
Soluble factors	β-chemokines, α-defensins, IFN-α and other unknown non-MHC-restricted soluble factors are all able to inhibit viral replication	[174-176]
APOBEC3 cytidine deaminase	Potent innate cellular factor against viral infection	[177]
Cytotoxic T-cell activity	Highly activated and broad HIV-specific CTLs are associated with low plasma viraemia in LNTPs	[178]
NK cell activity	Kill virus-infected cells and bridges the innate and adaptive cell immunity through the production of pro-inflammatory cytokines	[179]
Mucosal responses	HIV-1-specific IgA, CD4+ and CD8+ CTLs that control viral entry and dissemination at port of entry	[180-182]
Virus factors		
Tropism	Transition between T- and M-tropic virus co-receptor usage affects pathogenicity	[183-185]
Variability and escape mutants	Host pressure might induce the emergence of pathogenic viral strains	[186-188]
Attenuation of the phenotype	Natural deletion frequently within the *nef* gene might slow disease progression and establish chronic infection	[189]
Subtypes	Different subtypes are associated with different virulence and transmissibility	[190,191]

CTL: Cytotoxic T lymphocyte; HLA: Human leukocyte antigen; LTNP: Long-term non-progressor; NK: Natural killer.

1.1 Natural infection and host determinants

The principal manifestations of HIV infection are the dysregulation of the immune system and the depletion of CD4+ T cells. However, there are a few exceptions. In fact, at one end of the spectrum of natural infection there are individuals, defined as long-term non-progressors (LTNPs), who are able to mount effective antiviral responses capable of controlling viral replication and progression to disease. These infected persons remain clinically healthy for a long period of time with low to undetectable plasma viraemia and a minimal loss of CD4+ T cells in the absence of any antiretroviral therapy. Furthermore, there are individuals, defined as multiply exposed individuals (MEUs), who include HIV-discordant couples having unprotected sex, sex workers and healthcare workers, who have remained HIV seronegative for years, despite repeated exposures to the virus, suggesting that resistance to infection may occur in the apparent absence of HIV-specific immune responses, as commonly determined by routine serology testing. Results from immunological and genetic studies performed on LTNPs and MEUs, as well as data from acutely infected patients, have helped to elucidate some of the potential mechanisms by which control of infection may occur (**Table 1**). It is now clear that both host and viral factors contribute to the outcome of the infection. Understanding the mechanisms of the 'resistance to infection' seen in MEU or of the 'lack of progression to disease', as observed in LTNP is of critical importance for the development of preventative or therapeutic vaccines, respectively.

1.2 Animal models of HIV infection

Animal models represent a tool to study the dynamics of immune responses, the pathogenesis for many infectious agents as well as tumour-associated diseases. Mice and rats represent the majority of small animals used in medical research. Their small size and low cost make them ideal for laboratory experiments, but they are not permissive to HIV replication and past efforts to circumvent this resistance have failed. Present approaches include: i) further development of the hu-SCID mice model; ii) establishment of HIV-1 models of human disease through modifications of the genetic background of mice and rats [2-4]; and iii) the generation of pseudoviruses containing HIV-1 genes able to replicate efficiently in conventional mice or rats [5-8].

Ideally, the human transgenic (CD4/CCR5) rabbit should represent a better model as HIV-1 infection in rabbit recapitulates some aspects of HIV-1 infection in humans [9]. However, adaptation of HIV to a heterologous host may lead to selection of variants with unknown and potentially irrelevant properties.

Thus, although these small animal models have contributed to a better understanding of the safety, immunogenicity and efficacy of vaccine approaches, as well as the effects of antiviral drugs, none of them is an ideal model for HIV/AIDS, thus forcing the scientific community to use non-human primate models.

Chimpanzees can be infected by HIV and some pathogenic effects were observed *in vivo* [10]. However, the high cost, low number of available animals and international restrictions

Table 2. Summary of relevant (safety, immunogenicity, efficacy) preventative vaccine approaches based on structural and non-structural HIV/SIV antigens combinations in non-human primate models (for more detailed, completed and updated preclinical trials see also [401])

Immunisation regimen			Adjuvant/ immunoadjuvant	Route	Challenge		
Immunogen	Priming immunogen	Booster immunogen			Virus	Protection from infection and/or from disease*	Ref.
HIV-1 Tat	6 or 10 µg of protein (×8)	16 µg of protein in ISCOMs (×1)	RIBI, Alum, ISCOMs or none	s.c./i.m. or i.d.	$SHIV_{89.6P}$ (MID_{50} 10, i.v.)	Yes (5/7)	[82]
SIV Tat and Rev	SFV $1 \times 10^{8-9}$ $TCID_{50}$ (×2)	MVA $1 \times 10^{8-9}$ $TCID_{50}$ (×2)	None	i.m.	SIV_{mac32H} (50 MID_{50}, i.v.)	Yes (2/2)	[80]
HIV-1 *tat*	0.2 or 1 mg of DNA (×8)	16 µg of protein in ISCOMs (×1)	Bupivacaine, ISCOMs	i.m. or i.d./i.m.	$SHIV_{89.6P}$ (10 MID_{50}, i.v.)	Yes (3/5)	[83]
SIV Tat and Rev	SFV $1 \times 10^{8-9}$ $TCID_{50}$ (×2)	MVA $1 \times 10^{8-9}$ $TCID_{50}$ (×2)	None	i.m.	SIV_{mac32H} (50 MID_{50}, i.v.)	Yes (2/4)	[79]
HIV-1 Tat	100 µg of protein (×3)	100 µg of protein (×1)	IFA (Seppic ISA-51)	i.m.	$SHIV_{89.6P}$ (30 MID_{50}, i.v.)	Yes (2/8)	[88]
HIV-1 Tat toxoid	100 µg of protein (×3)	100 µg of protein (×1)	IFA (Seppic ISA-51)	i.m.	$SHIV_{89.6P}$ (30 MID_{50}, i.v.)	Yes (2/8)	[88]
HIV-1 Tat	0.8 mg of DNA (×4)	SeV 1×10^8 IU (×1)	None	i.m., gene gun, i.n.	$SHIV_{89.6P}$ (50 MID_{50}, i.v.)	Yes (3/3)	[87]
SIV *Env, Gag, Pol, Tat, Rev, Nef*	MVA 1×10^8 pfu (×1)	MVA 1×10^8 pfu (×2)	None	i.m.	SIV_{mac251} (50 MID_{50}, i.v.)	Yes (2/3)	[192]
HIV-1 Env, Rev, and SIV Gag, Pol	0.2 mg of each DNA (×2)	0.5 mg of each DNA (×2 or ×3)	Bupivacaine-HCl	i.m.	Sequential challenges SHIVIIIB (≥ 10 MID_{50}, i.v.) SIV_{mac239} (10 MID_{50}, i.v.) $SHIV_{89.6P}$ (10 MID_{50}, i.v.)	Yes (3/8) Yes (3/3) Yes (3/3)	[193]
SIV Gag, Pol, Vif, Vpx and Vpr plus HIV-1 Env, Tat and Rev	0.25 – 2.5 mg of DNA (×2)	MVA 2×10^8 pfu (×1)	None	i.d., i.m.	$SHIV_{89.6P}$ (NA, i.r.)	Yes (24/24)	[194]
SIV Env/Rev plus Gag	5×10^8 Ad5hr for each recombinant, for each route (×2)	SIV 100 µg gp120 protein (×2)	QS-21	o., i.n., i.m.	SIV_{mac251} (NA, i.r.)	Yes (8/12)	[195]

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Immunisation regimen			Adjuvant/ immunoadjuvant	Route	Challenge		
Immunogen	Priming immunogen	Booster immunogen			Virus	Protection from infection and/or from disease*	Ref.
HIV Nef–Tat fusion protein and SIV Nef	20 µg (×2)	20 µg (×1)	AS02A	i.m.	$SHIV_{89.6P}$ (5 – 50 MID_{50}, i.v.)	No (0/4)	[196]
HIV Nef–Tat fusion protein, SIV Nef and Env proteins	20 µg (×2) Env 100 µg (×2)	20 µg (×1), Env 100 µg (×1)	AS02A or AS06	i.m.	$SHIV_{89.6P}$ (5 – 50 MID_{50}, i.v.)	Yes (4/8)	[196]
SIV *Tat, Rev, Nef* and *Gag*	32 – 64 µg of each DNA (×6)	MVA 1×10^8 IU/ each Ag (×2)	Gold particles	i.e., o., i.d., i.n., i.v.	SIV_{mac239}, (10 MID_{50}, i.r.)	No (0/3)	[196]
SIV *Gag*, *Pol*, *Tat*, *Rev*, *Nef* and *Env*	100 µg of DNA/each Ag (×1)	SFV10^8 IU/ each Ag (×2) plus MVA 1 × 10^8 $TCID_{50}$/ each Ag (×1)	None	i.d., s.c., i.m.	SIV_{mac251} (50 MID_{50}, i.r.)	Yes (3/4)	[197]
Multigenic vaccine (SIV_{mac239} Gag and Pol, HIV-1 Tat, Rev, Vpu, and the 5′ portion of Env)	1 mg of DNA/each Ag (×2) 1 mg of DNA/each Ag (×1) 1 mg of DNA/each Ag (×1) 1 mg of DNA/each Ag (×2)	FPV 5 × 10^7 pfu/ each Ag (×1) FPV 5 × 10^7 pfu/ each Ag (×1) 1 mg of DNA/ each Ag (×2) FPV 5 × 10^7 pfu/ each antigen (×1)	None None None IFN-γ	i.m. i.m. i.m. i.m.	$SHIV_{mn229}$ (1 × 10^5 $TCID_{50}$, i.r.)	Yes (6/6) No (0/6) No (0/6) No (0/6)	[198]
Multigenic vaccine (SIV_{mac239} Gag and Pol, HIV-1 Tat, Rev, Vpu, and the 5′ portion of Env)	1 mg of DNA (×3)	FPV–SIV Gag/Pol plus FPV–HIV–AE Env 0.5 – 1 × 10^8 pfu (×2)	DOTAP	i.m., i.n., i.m. and i.n., or i.m. and i.r.	$SHIV_{SF162P3}$ (6 × 10^3 $TCID_{50}$, i.vag.)	Yes (9/14)	[199]
SIV_{mac239} Gag, Tat, Rev and Nef	5 mg of DNA (×3)	1 × 10^{11} pfu of Ad (×1)	CRL-1005/BAK	i.m.	Repeated low dose SIV_{mac239} (300 $TCID_{50}$)	Yes (8/8)	[200]

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Immunogen	Priming immunogen	Booster immunogen	Adjuvant/ immunoadjuvant	Route	Virus	Protection from infection and/or from disease*	Ref.
HIV-1 Tat	10 – 40 μg protein (×4)	20 μg protein (×1)	Polyphosphazene, IFA	i.d., i.m.	$SHIV_{89.6P}$ (2500 $TCID_{50}$, i.r.)	Yes (2/4)	[201]
HIV-1 Tat toxoid ± Env (vaccinia virus and protein)	Tat 20 – 60 μg (×4), or Tat 20 – 80 μg (×4) plus vaccinia virus encoding Env	Tat 40 μg (×1) or Tat 40 μg plus Env 100 μg (×1)	Polyphosphazene, IFA	i.d., i.m.	$SHIV_{89.6P}$ (2500 $TCID_{50}$, i.r.)	Yes (6/12)	[201]
HIV-1 Tat peptides (2), each conjugated to diphtheria toxoid	0.1 mg of each peptide (×1)	0.1 mg of each peptide (×3)	CFA, IFA	i.m.	$SHIV_{33}$ (50 MID_{50}, i.v.)	NA (low pathogenicity SHIV)	[202]
HIV-1 Tat peptides (2), each conjugated to diphtheria toxoid	0.5 mg of each peptide (×1)	0.5 mg of each peptide (×2)	CFA, IFA	i.m.	$SHIV_{33A}$ (200 MID_{50}, i.v.)	No (0/7)	[202]
HIV-1 Tat	SFV-*tat* 1 × 10⁸ $TCID_{50}$ (×2)	MVA 1 × 10⁸ $TCID_{50}$/each Ag (×2)	None	s.c., i.m.	$SHIV_{BX08}$ (10 MID_{50}, i.r.)	Yes (1/4)	[203]
HIV-1 Tat and Rev	SFV 1 × 10⁸ $TCID_{50}$/ each Ag (×2)	1 × 10⁸ $TCID_{50}$ MVA-*tat* (×2)	None	s.c., i.m.	$SHIV_{BX08}$ (10 MID_{50}, i.r.)	Yes (1/4)	[203]
HIV-1 *Tat* and *Rev*	1 mg of DNA/ each Ag (×2)	0.25 mg DNA/ each Ag (×2)	IL-12	i.d., i.m.	$SHIV_{BX08}$ (10 MID_{50}, i.r.)	Yes (2/4)	[203]
IV *Nef*	0.1 mg of each plasmid (×4)	0.5 mg of each plasmid (×5)	None	i.m.	SIV_{mac251} (NA, i.r.)	Yes (2/2)	[204]
SIV *Nef*, *Vpr* and *Vpx*	0.1 mg of each plasmid (×4)	0.5 mg of each plasmid (×5)	None	i.m.	SIV_{mac251} (NA, i.r.)	No (2/2)	[204]
HIV-1 Tat	0.5 mg of DNA (×3)	25 μg of protein (×3)	Bupivacaine, methyl paraben, ISCOMs	i.m.	$SHIV_{89.6P}$ (50 MID_{50}, i.v.)	No (0/4)	[205]

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Immunisation regimen			Adjuvant/ immunoadjuvant	Route	Challenge		
Immunogen	Priming immunogen	Booster immunogen			Virus	Protection from infection and/or from disease*	Ref.
HIV-1 Tat plus HIV Env and SIV Gag	0.5 mg of each DNA (×3)	25 µg of each protein (×3)	Bupivacaine, methyl paraben, ISCOMs	i.m.	SHIV$_{89.6P}$ (50 MID$_{50}$, i.v.)	Yes (4/4)	[205]
SIV Env/Rev, Env/Rev plus Gag, Env/Rev plus NefΔ_{1-13} or Env/Rev plus Gag plus NefΔ_{1-13}	5 × 10^8 Ad5hr for each recombinant, for each route (×2)	SIV 100 µg gp120 protein or SIV peptomer (×2)	MPL-SE or none	o. and i.n., i.t., i.m.	SIV$_{mac251}$ (10 MID$_{50}$, i.r.)	Yes (11/39)	[161]
HIV-1 Tat	1 × 10^{11} Ad5 (×2)	1 × 10^{11} Ad5 (×1)	None	i.m.	SHIV$_{89.6P}$ (50 MID$_{50}$, i.v.)	No (0/4)	[86]
HIV-1 Tat	100 µg protein (×1)	100 µg protein (×3)	Montanide ISA-720	s.c.	SHIV$_{BX08}$ (NA, i.r.)	Yes (7/7)	[85]
SIV$_{mac239}$ Gag–Env, Pol, Vif–Nef and Tat–Vpx	2 mg of DNA (×3)	3.75 × 10^{11} rAd5 (×1)	IL-12	i.m.	SIV$_{mac251}$ (50 MID$_{50}$, i.r.)	Yes (3/4)	[206]
SIV Env/Rev, Env/Rev plus Gag, Env/Rev plus NefΔ, or Env/Rev plus Gag plus NefΔ	5 × 10^8 Ad5hr for each recombinant, for each route (×2)	SIV 100 µg gp120 protein or SIV peptomer (×2)	MPL-SE or none	o. and i.n., i.t., i.m.	SIV$_{mac251}$ (10 MID$_{50}$, i.r.)	Yes (8/11)	[160]

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drastically limit their usage. Attempts to productively infect *Macaca nemestrina* with HIV-1 resulted in a low level of virus replication in the absence of pathogenetic effects [11]. In contrast, both *M. nemestrina* and baboons are readily infected with pathogenic HIV-2 isolates [12], which are much closer to simian human immunodeficiency virus (SIV) than to HIV-1.

Of interest, African green monkeys and Sooty mangabeys, which are naturally infected with SIV (SIVagm and SIVmn, respectively), show no evidence of pathogenic effects despite high levels of viral replication indicating virus–host

adaptation [13]. These models represent valuable tools to understand the dynamics of virus replication and the impact of host immune responses on disease progression.

In contrast, the experimental infection of macaque species such as *M. mulatta*, *M. nemestrina* and *M. fascicularis* with SIV induces high levels of plasma viraemia, resulting in a spectrum of pathological responses similar to those observed in HIV-1-infected individuals. For these reasons, the macaque–SIV model appears to be more appropriate to help elucidate the pathogenetic mechanism(s) of HIV-1 infection in

humans and to characterise the complex and multifaceted natural and adaptive immune responses to infection [14,15]. Therefore, macaques represent an important model for the evaluation of safety, immunogenicity and efficacy of vaccine candidates, including the identification of immune correlates of protection as well as the genetic factors impacting on pathogenesis and on vaccine effects. Despite the phylogenetic vicinity, SIV has very low sequence homology and crossreactivity with HIV-1. To overcome this issue and to directly test HIV-1 vaccines in an animal model, chimeric simian/human immunodeficiency viruses (SHIV) have been generated by inserting HIV genes into the genome of SIV_{mac239}. The pathogenicity of these chimaeras is either very high ($SHIV_{89.6P}$, CXCR4-tropic) [16] or moderate ($SHIV_{sf162P3}$, CCR5-tropic) [17], indicating that further development and refinement of the SHIV–macaque model is needed to mimic the natural course of HIV infection. The relevance of both the SIV and SHIV models is under debate because, in the experimental infection, cell-free rather than cell-associated virus is used at doses that are much higher than those estimated to be transmitted in the course of natural infection with HIV, possibly hampering recognition of potentially protective vaccines [18,19].

Besides these limits, the SIV and SHIV macaque models have provided important insights into the safety, immunogenicity and efficacy of a number of vaccine approaches. In fact, vaccines based on live attenuated SIVs have consistently protected monkeys against single or repeated challenges with homologous or heterologous pathogenic viral strains, demonstrating that induction of protective immunity is indeed feasible [20,21]. However, as ethical concerns prohibit the use of attenuated virus in human [22], a number of approaches aimed at mimicking attenuated live virus vaccines have been widely tested in non-human primate models.

Many attenuated bacterial and viral vectors and naked DNA expression constructs have been or are being used in these preclinical models [401]. Similarly, numerous novel adjuvants, although not approved yet for use in humans, have been tested for their ability to enhance T- and/or B-cell immune responses (**Table 2**). Although some of these approaches have been effective in inducing immune responses (predominantly T-cell mediated), and to a certain degree curb viral replication and delay progression to overt disease in macaques, none has so far matched the full protection mediated by live attenuated virus vaccines.

1.3 Virus variability

In spite of a number of efforts to develop an effective vaccine against HIV/AIDS, there is still no clear evidence of how this should be accomplished. A major hurdle in the development of vaccines against HIV/AIDS is our lack of knowledge of the immune correlates of protection, the protective immune responses that must be elicited in order to prevent infection and/or disease onset. Without knowledge of the critical antigens or effective defence mechanisms, the strategies applied in vaccine development mostly rely on a 'trial and error'

approach and may be fundamentally flawed. One of the most critical challenges is the high genetic variability of HIV. As a result of errors generated by the reverse transcriptase and the lack of proof-reading functions associated with the polymerase, vast heterogeneity occurs throughout the viral genome with 'hotspots' especially in glycoprotein (gp)120. The gp120 viral envelope protein is highly glycosylated, potently immunogenic and forms trimers via non-covalent bonds with the cellular membrane-spanning gp41 envelope protein. The oligomeric envelope spikes decorating the viral surface are the main target for neutralising antibodies. Escape mutants arise not only by amino acid substitutions or conformational changes affecting the neutralising epitopes, but also by changes in the glycosylation sites [23,24], posing an additional obstacle to the realisation of sterilising vaccines. Furthermore, because of inefficient or defective assembly, gp120 monomers, which display many type-specific or non-neutralising epitopes, are continuously released by productively infected cells, thus acting as a decoy for the immune system and potentially representing an effective mechanism of immunoevasion [25]. To partially circumvent these problems, strategies aimed at inducing cellular immune responses capable of containing virus replication and blocking disease onset have been developed [26]. Indeed, such strategies induced cellular responses that in many cases were able to control viral replication down to undetectable levels and/or to delay the clinical progression of the infection, but not to provide the level of protection necessary to prevent the appearance of viral escape mutants [27,28].

1.4 Virus-associated cellular proteins

As a further complication, the HIV envelope contains not only viral proteins, but also components derived from the host's cellular plasma membrane or from the intracellular compartments of the endosomal network [29]. Many molecules (lipid rafts, MHC class I and II, CD80, CD86, CD28, inflammatory cell adhesion molecule-1 and -3, CD44, complement proteins, cardiolipin, etc.) have been detected on the surface of HIV virions, most of which retain their biological activities. Thus, the selective acquisition of specific cellular proteins by the envelope of lentiviruses (HIV, SIV) might affect virus infectivity and tropism, and elicit T-cell signalling in bystander cells, thus increasing the susceptibility to infection and disturbing immune cell functions [30]. The impact of these 'cellular contaminants' on the immune response was revealed by a study in which macaques were vaccinated either with non-infected human cells or with whole-inactivated virus grown on human cells. Regardless of the vaccine received, all macaques were 'protected' following challenge with a SIV strain propagated *in vitro* in the same cells used to prepare the whole-inactivated virus vaccine. However, when these 'protected' monkeys were superinfected with a virus strain grown on monkey peripheral blood mononuclear cells, they all became infected, thus indicating that protection was likely due to the immune

Table 3. Regional HIV and AIDS statistics and features as of December 2006.

	Adults and children living with HIV (mean)	Adults and children newly infected with HIV (mean)	Adult prevalence %	Per cent of HIV+ adults (age 15 – 49) who are female (%)	Adult and child deaths due to AIDS (mean)
Sub-Saharan Africa	24.7 million	2.8 million	5.9	59	2.1 million
North Africa and Middle East	460,000	68,000	0.2	48	36,000
South and Southeast Asia	7.8 million	860,000	0.6	29	590,000
East Asia	750,000	100,000	0.1	29	43,000
Oceania	81,000	7100	0.4	47	4000
Latin America	1.7 million	140,000	0.5	31	65,000
Caribbean	250,000	27,000	1.2	50	19,000
Eastern Europe and Central Asia	1.7 million	270,000	0.9	30	84,000
North America	1.4 million	43,000	0.8	26	18,000
Western and Central Europe	740,000	22,000	0.3	28	12,000
Total	39.5 million	4.3 million	1.0	48	2.9 million

Modified from 'AIDS Epidemic Update 2006' [402].

response elicited against the cellular rather than the viral components of the vaccine [31,32].

1.5 Mucosal immunity

As for many common infectious agents, HIV gains access to the host through mucosal membranes, both in the horizontal and vertical modalities of transmission. Studies of SIV infection in macaques indicated that, regardless of the route of infection, the gastrointestinal and vaginal mucosae represent the major site of HIV entry, replication and amplification, and the initial site of CD4+ T-cell depletion [33]. Although mucosal sites contain specialised immune cells including antigen-presenting cells (APCs) such as dendritic cells (DCs), which are involved in the induction and regulation of antiviral immunity, HIV can clearly overcome the first line of immune defence and establish a productive infection [34]. Nonetheless, experimental evidence indicates that mucosal immune responses to HIV may contribute to the low rate of transmission observed and appear to contain, to some extent, virus replication. For example, secretory IgA inhibit virus assembly and intracellular release and play an important role in inhibiting HIV transmission via the mucosal route, as clearly demonstrated in multiple-exposed females [35,36]. Furthermore, multiple rectal exposures to low doses of SIV induced MHC class I-restricted cytotoxic responses that protected against a mucosal challenge with a heterologous virus [37]. Similarly, studies in the macaque model provided insight into the protective role of high-avidity cytotoxic responses generated at the mucosal level. A peptide-prime/poxviral-boost vaccine,

which induced mucosal CD8+ cytotoxic T lymphocytes (CTLs), controlled the dissemination of intrarectally administered pathogenic SHIV. Protection correlated with mucosal rather than with systemic CTLs, and particularly with the level of high-avidity mucosal CTLs elicited by intrarectal vaccine administration [38]. A number of studies demonstrated that mucosal immunisation can elicit both mucosal and systemic immunity and that mucosal immune responses (mucosal IgA and IgG responses as well as mucosal CD8+ CTLs) may contribute to control viral replication and dissemination more effectively than those elicited by systemic immunisation [39,40]. However, our present understanding of mucosal immunology, as well as of early events of HIV interactions at the mucosal level, including the role played by non-structural HIV proteins, is still inadequate [41,42]. In fact, identification of the right vaccine candidate(s), as well as of adjuvant and immunomodulators promoting long-term mucosal immune responses is critical for the development of a preventative vaccine for adults and children.

2. Medical need and existing treatment

2.1 AIDS epidemic

According to recent estimates, 39.5 million people are living today with HIV infection. In 2006, 4.3 million people were newly infected, of which 530,000 were children < 15 years of age. During the year 2006, 2.9 million people died as a result of HIV/AIDS (**Table 3**). Sub-Saharan Africa and Asia remain the regions most devastated by the epidemic and rates of
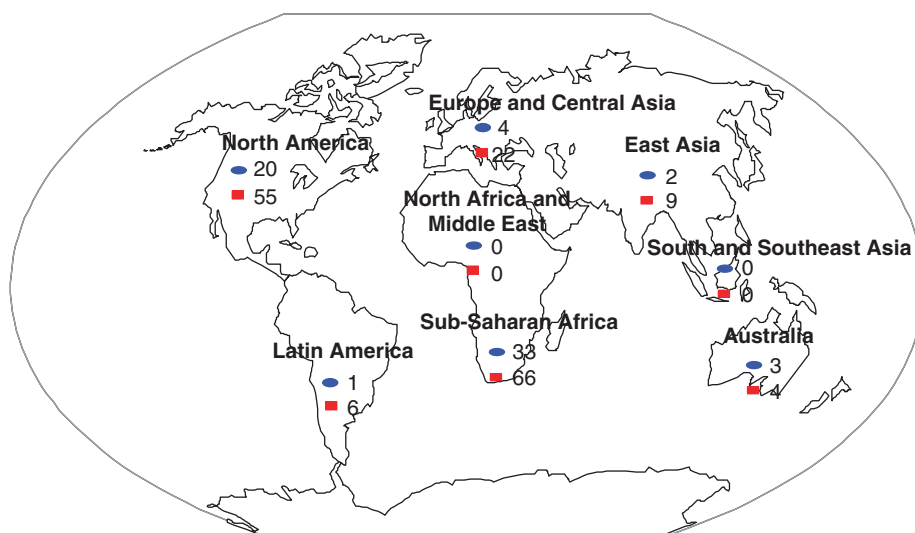
Figure 1. Geographical distribution and number of new (circle) and old (square) preventative or therapeutic clinical trials around the world as reported in [403] and [404].

infection continue to increase. Dramatically, females and adolescents are placed at the centre of the AIDS epidemic as a high-risk population for HIV infection. The effect of HIV infection on individuals and communities is socially and economically devastating and transcends gender differences, age, sexual orientation, socio-economic status and nationality. Thus, the social and economic impact of the HIV/AIDS pandemic is unprecedented, and may lead to political instability, misguided market reforms and famine.

2.2 Therapeutic approaches with antiretroviral drugs

The introduction almost 10 years ago of HIV-1 protease inhibitors in addition to non-nucleoside reverse transcriptase inhibitors offered the opportunity to implement combination therapy, termed highly active antiretroviral therapy, which combines metabolic inhibitors targeting different viral enzymes, leading to a significant reduction of viral load, with partial restoration of $CD4^+$ T-cell numbers, immune functions and a substantial delay of disease progression [43]. More recently, a new family of antiretroviral compounds, which inhibits different steps of the viral entry process including: i) gp120 attachment to the CD4 receptor; ii) binding of gp120 to CCR5 or CXCR4 coreceptors; and iii) fusion of viral and cellular membranes [44,45], has been introduced and appears to be superior to the previous generation of drugs and possibly effective against resistant virus strains. Because of their mechanisms of action, these compounds are also being evaluated as candidate microbicides to prevent HIV mucosal transmission [46]. Studies in the non-human primate models have provided evidence of the effectiveness of highly active antiretroviral therapy in preventing or delaying the establishment of persistent infection [47,48]. However, the beneficial effects of any of these drugs are lost following treatment

interruption. Thus, as drug therapy does not appear to qualitatively modify immune responses [49-51], there is a growing interest in therapeutic approaches combining antiretroviral treatment with vaccination.

Although the development of antiviral drugs has had a dramatically beneficial effect on those HIV-infected individuals who have access to therapy, it has had little impact on the global HIV epidemic. Drug toxicity, emergence of resistant virus strains and the absence of a correct education to achieve full compliance to therapy, render drug therapy difficult to implement and to manage, particularly in poor endemic areas. Finally, it is still unclear whether these therapeutic treatments have the potential to eradicate the virus present in cryptic body compartments and to block mother-to-child, including milk-borne, transmission of infection [52].

Therefore, the development of novel therapeutic approaches remains a priority and an urgent medical need in order to gain successful control of the HIV epidemic.

2.3 Preventative vaccine

Although the correlates of protective immunity are still elusive, there is substantial evidence that broadly neutralising antibodies, T helper (T_H) and cytotoxic activities, innate immunity, and long-lived memory T- and B-cell responses are all important for protection and control of disease progression [53-57]. Consequently, preventative vaccine strategies able to elicit humoral and cell-mediated responses both at systemic and mucosal sites have been developed, including: i) prime–boost regimens of immunisation; ii) native or modified viral proteins; iii) recombinant viral or bacterial vectors expressing viral proteins; iv) new adjuvants; v) inclusion of immunomodulators such as cytokines, chemokines or costimulatory molecules; and vi) use of autologous DCs

pulsed with viral antigens. Most of these vaccine approaches have been shown to be safe, immunogenic and, to some extent, protective in preclinical models and have, therefore, been tested in humans. As of April 2006, there were > 30 preventative AIDS vaccine candidates in early stages of human clinical trials [403,404] in different countries around the world (**Figure** 1). Notably, despite relentless efforts, no preventative vaccine is yet available to combat the HIV/AIDS epidemic.

3. Therapeutic class review

Antiretroviral treatment requires caution and strict monitoring of drug toxicity as well as virus sensitivity to therapy and compliance. This is not yet easily achieved in the developing world. Compliance with therapy regimens is difficult, and the efforts of developing countries to modify the behaviour and culture of their populations through intervention programmes of the National Public Health Plans have had only a modest, although measurable, impact. In addition, the emergence of multi-drug resistant HIV-1 strains, due to virus adaptation to therapeutic pressure, already represents a major obstacle in long-term treatments. Therefore, prevention of transmission through the development of a vaccine against HIV/AIDS may conceivably be the most effective way to stop the spread of HIV infection. However, there is no HIV/AIDS vaccine on the market and because vaccine development is a high-risk project, only a few companies are working on it.

4. Current research goals

4.1 Vaccine based on structural HIV-1 protein: Env
For many years, HIV vaccine strategies have mainly targeted the structural protein Env and, to a lesser extent, Gag. However, few have provided significant protective immunity in non-human primate models and, most importantly, results from clinical trials, including the first Phase III trial (AIDS-VAX, VaxGen) based on monomeric gp120 [58], have been largely disappointing.

In fact, immunisation with Env-induced antibodies able to neutralise: i) homologous, but not heterologous, viruses in a preclinical challenge model [59]; and ii) lab-adapted, but not primary, virus isolates [60]. This represents a major problem as during their life vaccinated individuals are likely to encounter viral subtypes with gp120 different from that used for vaccination. Thus, vaccine approaches capable of inducing cross-clades neutralising antibodies needed to achieve sterilising immunity are not available yet.

In this regard, HIV-1 Env oligomers, consisting of trimers of gp120 fused with the ectodomain of gp41, are superior to the monomeric gp120 for eliciting strong humoral responses directed toward conformational epitopes [61,62], as well as neutralising antibodies against both T-cell line adapted (X4) and selected primary HIV isolates (R5 and X4) [63,64]. However, because of its conformation and of the presence of glycan

residues, the conserved neutralising epitopes in the native gp120 are not accessible or recognised by the immune system [65,66].

Experimental data support the hypothesis that cross-clade and broadly neutralising antibodies can be induced against epitopes present in the V3 region of Env [67]. Indeed, an anti-V3 humanised antibody has been shown to block the *ex vivo* generation of primary HIV-1 quasispecies in peripheral blood mononuclear cell cultures from infected individuals and to protect monkeys from infection with SHIV, thus providing sterilising immunity [68].

In principle, at least two series of approaches can be envisaged to induce protective antibodies: i) to prepare a vaccine containing Envs from different clades; and ii) to introduce modification in the gp120/160 molecule to induce exposure of otherwise cryptic neutralising epitopes [23,24,69]. In preclinical models, immunisation with polyclonal Env induced antibodies with limited ability to neutralise primary isolates or to protect monkeys [70]. Similarly, vaccination with Env glycoproteins in which an intermolecular disulfide bond between gp120 and gp41 subunits had been introduced to stabilise Env conformation failed to induce potent cross-neutralising and fusion-blocking antibodies [71]. In another approach, vaccination with monomeric gp120 or oligomeric gp140 molecules covalently bound to CD4 has been reported to induce, in macaques, crossreactive antibodies [72] whose neutralising activity remains to be confirmed.

Perhaps the most interesting results come from a novel variant of trimeric Env containing a deletion in the second variable loop (ΔV2-Env), which was developed and tested in preclinical models [73,74]. Vaccination with ΔV2-Env protein induced cross-clade neutralising antibody in rabbits using a DNA-prime/protein-boost regimen, and contained infection in monkeys following virus challenge [64,75].

Thus, modified Envs appear to represent a substantial advancement in the design of an effective Env-based vaccine.

4.2 Vaccine based on non-structural HIV-1 proteins: Rev, Tat, Nef
The formidable obstacles encountered in the preparation of an anti-Env vaccine providing sterilising immunity prompted the scientific community to consider alternative strategies. Thus, novel approaches, based on the new concept of 'reverse vaccinology' have been developed with the aim of blocking virus replication and disease onset by targeting non-structural HIV regulatory genes, which are essential for replication and infectivity [76]. Control of virus replication, achieved in the absence of sterilising immunity, should provide protection from disease progression and reduce virus transmission to healthy individuals.

Rev, Tat and Nef are the first HIV proteins expressed early after infection and are essential for virus replication. In addition, Tat and Nef are also found extracellularly and, therefore, represent ideal targets for vaccine strategies [76]. Besides their effects on the virus life cycle, these non-structural proteins are immunogenic and exert multiple

dysregulatory effects on bystander cells, either directly or indirectly, aimed at facilitating immune cell recruitment and activation to increase viral infection and transmission. Tat, Rev and, to a lesser degree, Nef proteins are highly conserved in their immunodominant regions encompassing B and T (including CTL) epitopes, a very desirable feature for the generation of a vaccine that has to face major intra- and interclade variability. Concerning Tat, sera from Italian, Ugandan and South African patients who are mainly infected with A, B, C and D and, to a lesser extent, F and G HIV-1 subtypes, recognise the BH-10 Tat protein to a similar extent (i.e., prevalence and titres of anti-Tat antibodies) [77,78].

In preclinical models, vaccination with Tat and Rev either alone or in combination with structural antigens efficiently controlled viral replication following challenge with pathogenic SIV or SHIV (Table 2). In the SIV–macaque model, vaccination with Tat and Rev elicited more effective CTL responses than immunisation against the structural Gag and Pol proteins, underscoring the importance of immuno-targeting early viral proteins [79], and protected macaques from challenge infection with a pathogenic SIV [80]. Furthermore, vaccination of macaques with a polyprotein including Rev, Tat and Nef induced cellular responses to all gene products [81]. In cynomolgus monkeys, vaccination with a biologically active Tat protein or *tat* DNA was safe, elicited a broad, specific immune response and, more importantly, dramatically controlled viral replication and blocked disease progression in vaccinated monkeys challenged with the highly pathogenic SHIV89.6P [82,83,301]. Of note, 'protection' was prolonged and no residual virus hidden in resting cells was detected in any of the protected macaques either in plasma or lymph nodes, following two boosts with tetanus toxoid, a stimulus known to activate T cells and induce virus replication. Long-term 'protection' correlated with the presence of high and stable humoral and cellular (CD4 and CD8 T cell) responses against Tat [84]. In the macaque SHIV model of infection, immunisation with a synthetic Tat protein (Tat Oyi) elicited protective anti-Tat antibodies, whereas in rabbits it induced cross-clade anti-Tat antibodies [85]. In other settings Tat vaccines did not provide significant protective immunity and escape mutants have been observed [86-89]. Whether these apparently conflicting results are due to the nature of the vaccine antigen (DNA versus protein, native versus inactivated Tat protein), the monkey species, the route of the administration, the antigen dose and schedule of immunisation, the adjuvant used or the virus challenge dose, remains to be elucidated.

Of note, humoral and cellular responses to Rev, Tat and Nef are present during the asymptomatic phase of infection and correlate with the non-progression to AIDS both in SIV-infected macaques and HIV-positive patients [90-95]. In particular, anti-Tat and anti-Nef antibodies are induced in the asymptomatic phase of infection and significantly correlate with the non-progression to AIDS both in HIV-1- or HIV-2-infected patients [94,96]. In asymptomatic HIV-1-infected individuals receiving antiretroviral therapy, intramuscular administration of plasmid DNA encoding Rev, Tat and Nef was safe and improved cellular (T$_H$ and CTL), but not humoral, immune responses [97]. Furthermore, in naive and HIV-1-seropositive patients the Tat protein inoculated either subcutaneously in the presence of Alum adjuvant or intradermally was safe and able to elicit humoral and cell-mediated immune responses (Table 4). More complex approaches combining structural and non-structural proteins have also been tested both in macaques and in humans (Tables 2 and 4). Although the vast majority of vaccines were well-tolerated, in many cases interference among the different antigens was observed, indicating that further optimisation of vaccine formulations and immunisation schedules are needed.

4.3 Mucosal immunity

Mucosal surfaces comprise the largest surface area of the human body and are the first line of defence against many pathogens. More than 90% of common infectious disease pathogens in humans gain access to the host through mucosal membranes both in horizontal (sexual intercourse) and vertical (child delivery and breastfeeding) transmission. The mucosal tissue contains several immune cells, including APCs, such as DCs, which are involved in the induction and fine regulation of antiviral immunity.

Vaccines almost certainly need to harness DCs to induce strong immune responses at mucosal sites. DCs are the most potent APCs for naive T lymphocytes. They efficiently present peptides of intracellularly synthesised antigens to CD8$^+$ T cells and are capable of presenting exogenously derived antigens both in the context of MHC class I (cross-presentation) and II molecules. Immature DCs resident in the periphery and in mucosal tissues are highly efficient samplers of extracellular antigens using several specialised mechanisms (endocytosis, phagocytosis, macropinocytosis and receptor-mediated capture) depending on the nature of the antigen. Toll-like receptors and C-type lectin membrane-bound molecules can recognise 'danger signals' associated with pathogens or foreign proteins and trigger DC activation and migration [98,99]. This is an important feature, which may be critical for the selection of appropriate delivery systems for vaccine antigens. 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 [100].

There is evidence that reinfusion of *ex vivo* antigen-pulsed DCs may have therapeutic applications against tumours and chronic SIV/HIV infection [101-103]. Although promising in its efficacy, the *ex vivo* pulsing of DCs and their reinfusion into the original donor is not practical and hardly feasible in developing countries. A much more feasible alternative is represented by designing vaccine formulations capable of properly targeting and stimulating DCs. In this regard, knowledge of the early events occurring at the mucosal level following viral exposure may facilitate the selection of the proper antigen(s) to target, as well as of adjuvant and delivery system.

Table 4. Phase I/II clinical trials evaluating preventative and therapeutic vaccines based on structural and non-structural HIV/SIV antigens.

Recipients	Immunogen	Dose (number of vaccine administrations)	Adjuvant	Route	Toxic effects*	Immune responses		Ref.
						Ab	**Cellular‡**	
HIV-positive or -negative individuals	HIV-1 Tat toxoid	50 – 100 µg	IFA	i.m.	None	+§	±	[207]
HIV-positive individuals	HIV-1 *tat*, *rev* or *nef* DNA	100 µg (×3)	None	i.m.	None	±	+	[208]
HIV-negative individuals	*env/rev* DNA	0.1 – 1 mg (×4)	None	i.m.	None	-	±	[209]
HIV-positive individuals	MVA expressing HIV-1 Nef	MVA 5×10^8 IU (×3)	None	s.c.	None	±	+	[145]
HIV-positive individuals	HIV *tat*, *rev* and *nef* DNA	100 µg (×1) plus 300 µg (×1) plus 600 µg (×1) for each antigen	None	i.m.	None	-	+	[97]
HIV-negative individuals	DNA and MVA encoding HIV-1 Gag and Env and HIV-1 (clade A) *env*, *gag*, *pol* and *nef* CTL epitopes (HIVA)	100 or 500 µg of DNA (×2) or MVA 5×10^7 pfu (×2), or 100 or 500 µg of DNA (×2) plus MVA 5×10^7 pfu (×1)	None	i.m., i.d.	None	-	+	[210]
HIV-1-naive and -infected individuals	GTU-Nef GTU multi-HIV	1, 20, 40 µg of DNA 2, 20, 100, 200, 2000 µg of DNA	None None	i.m/i.d i.m/i.d	None None	- -	+ +	[167]
HIV-1-exposed newborns and infants	HIV-1 gp120	vCP205 (ALVAC) $10^{6.08}$ TCID$_{50}$ or $10^{6.33}$ TCID$_{50}$ (×4)	None	i.m	None	+	+	[211]
HIV-positive individuals	MVA expressing HIV-1 Nef	MVA 5×10^8 TCID$_{50}$ (×3)	None	s.c.	None	+	+	[212]
HIV-negative individuals	HIV p24/p17 DNA and MVA or MVA	4 mg of DNA (×2) plus MVA10^8 (×1) or MVA10^8 (×2)	None	i.m. i.d i.d.	None None	NA NA	+ ±	[213]
HIV-1-naive and -infected individuals	HIV-1 Tat	7.5 µg (×5) 15 µg (×5) 30 µg (×5)	Alum/none Alum/none Alum/none	s.c/i.d s.c/i.d s.c/i.d.	None None None	+ + +	+ + +	Ensoli B, in preparation

*Toxic effects do not include mild local or systemic reactions.

‡Either T$_H$ response evaluated by lymphoproliferative assay, or CTL responses measured either by cytotoxic assay, or IFN-γ secretion/expression (ELISA, ELIspot, or intracellular cytokine staining).

§+, ≥ 50% responders, ±, < 50% responders.

Ab: Antibody (IgG, IgA) responses measured by ELISA; CTL: Cytotoxic T lymphocyte; ELISA: Enzyme-linked immunosorbent assay; gp: Glycoprotein; i.d.: Intradermal; IFA: Incomplete Freund's adjuvant; i.m.: Intramuscular; MVA: Modified vaccinia virus, Ankara strain; NA: Not available; s.c.: Subcutaneous; TCID$_{50}$: Tissue culture infectious dose 50.

For more detailed and updated description of ongoing trials see [403].

The development of delivery systems devoid of reactogenicity is an expanding field of research. Successful incorporation of proteins in poly(DL-lactide) and poly(DL-lactide-co-glycolide) biodegradable microparticles with respect to loading and encapsulation efficiency, as well as microparticle size and morphology, have been reported [104]. Of importance, mucosal administration of vaccine antigens encapsulated in microspheres elicited strong, long-term immune responses in mice [105,106]. Nevertheless, following encapsulation and release, DNA and proteins are easily degraded, leading to a significant reduction in vaccine efficiency. Thus, in more recent approaches antigen is bound to the surface of nano- and microparticles by means of charged molecules adsorbed on the particles' surface (i.e., cetyltrimethylammonium bromide and sodium dodecyl sulfate),
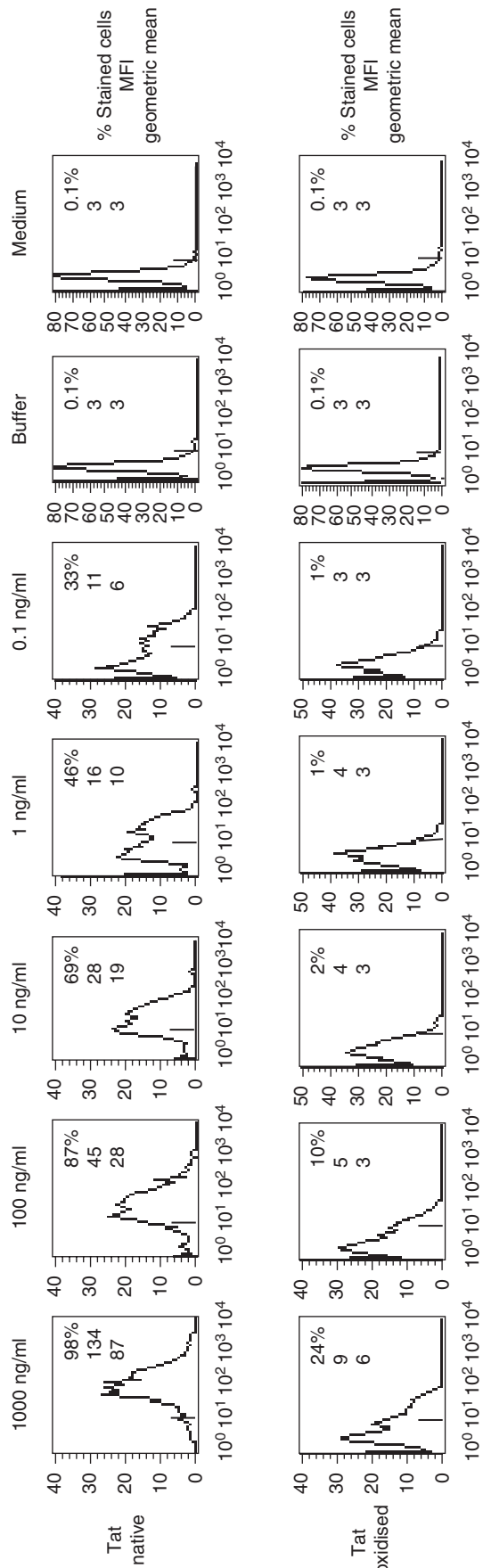
Figure 2. Native, but not oxidised, Tat is efficiently taken up by MDDCs. MDDCs were obtained from peripheral blood monocytes of healthy human donor and purified by incubation with anti-CD14-coated microbeads followed by sorting with a magnetic device. The purity of monocytes was always > 95%, as assessed by flow cytometry. MDDCs were incubated for 10 min with serial concentrations (0.1 – 1000 ng/ml) of the native Tat protein, medium or reconstitution buffer, respectively. Cells were then processed, washed, fixed and permeabilised, and the intracellular Tat content was evaluated by flow cytometry after staining with specific affinity-purified rabbit anti-Tat polyclonal antibody (or isotype control), followed by secondary fluorescein isothiocyanate-conjugated antirabbit antibody. Experiments were also performed with Tat oxidised (0.1 – 1000 ng/ml) by exposure to light and air for 18 h. Using this procedure, Tat loses its biological activity due to conformational changes, including multimerisation and aggregation of the protein with loss of the monomeric active form.

MDDC: Monocyte-derived dendritic cell; MFI: Mean fluorescence intensity.

which provide functional groups able to reversibly bind proteins/peptides, or covalently bind onto the particle surface (i.e., Eudragit and polyethylene glycol) [107,302,303].

5. Scientific rationale

5.1 Vaccine based on structural HIV-1 proteins: the Env antigen

A vaccine truly capable of preventing infection (i.e., providing sterilising immunity) should elicit broadly neutralising antibodies [56]. Ideally, antibodies should: i) block cell-free virus; ii) inhibit the Env-mediated interactions of HIV with cellular receptors and co-receptors, virus–host membrane fusion and virus budding; and iii) bind to complement proteins or Fcγ receptors on effector cells, thereby promoting opsonisation, complement-mediated lysis of viral particles and antibody-dependent cell-mediated cytotoxicity. HIV-1 infection induces polyclonal antibody responses to a large number of epitopes and it is quite evident that native gp120 in either the trimeric or the monomeric form is relatively inefficient at eliciting protective neutralising responses, while inducing very high titres of binding antibodies. Interestingly, studies on sera from HIV-infected individuals helped to identify potentially protective epitopes localised in the gp120 and gp41 proteins [69,108]. Indeed, pre- or postvirus exposure passive immunisation with cocktails of exceptionally potent neutralising monoclonal antibodies (F105, 2F5, 2G12 and b12) derived from infected patients protected non-human primates from intravenous or mucosal SHIV challenge [109,110]. This is in line with the growing consensus that the quality rather than the quantity of neutralising antibodies determines the possibility of achieving complete protection from infection. In this respect, the recent development of immunogens from subtype B and C, which are deleted in the V2 region (ΔV2-Env) represent an important advancement, as preclinical studies have shown that ΔV2-Env protein is immunogenic and able to induce cross-clade neutralising antibodies, as well as to significantly contain infection in monkeys challenged with the CCR5-tropic SHIVSF162P4 virus. Some of the protected macaques remained negative for plasma viral RNA, with stable numbers of CD4+ and CD8+ T lymphocytes, no signs of disease and no evidence of the emergence of escape viruses [74,75]. Furthermore, priming with a replication-competent adenoviral vector encoding HIV-1 Env followed by boosting with the oligomeric ΔV2-Env protein elicited in chimpanzees an antibody response with broad neutralising activity and cross-clade antibody-dependent cellular cytotoxicity [111,112].

5.2 Vaccine based on non-structural HIV-1 proteins: Rev, Tat and Nef

The HIV-1 transactivating factor Tat is a key viral regulatory protein produced very early after infection, even prior to HIV integration, is necessary for viral gene expression and plays a critical role in AIDS pathogenesis. Tat is released by acutely infected T cells in the extracellular milieu, binds to the extracellular matrix via interaction with heparan sulfate proteoglycans and enters neighbouring cells, altering their function regardless whether they are infected or not [113-116]. In fact, Tat has been found to modify both viral and cellular gene expression [114,117,118] and, although it does not appear to enter B- or T-cell blasts, induces the release of IL-6 and -10 from lymphoblastoid B cells [119] and differentially modulates the response of distinct B-cell subsets to polyclonal stimuli [120]. Furthermore, Tat has been shown to lower the activation threshold of naive T cells [121] to upregulate CCR5 and CXCR4 expression [122,123], to trigger activation-induced rather than Fas-L-induced apoptosis [124], and to downregulate the expression of CD127 on purified human T cells, inhibiting CD8 T-cell activity and function (proliferation, perforin synthesis and cytotoxic activity) [125].

Nef, a key regulatory protein of HIV-1 expressed early in the virus life cycle and released in the extracellular milieu, displays many biological activities including receptor downregulation, T-cell activation and cytoskeleton rearrangement [126,127]. Nef protein enters B cells and by 'hijacking' physiological feedback inhibitors in B cells, suppresses immunoglobulin class-switch and may contribute to the evasion of protective T-cell-dependent IgG and IgA responses [128,129]. In addition, by decreasing the surface expression of MHC class I on HIV-1-infected T cells, Nef interferes with the ability of CTLs to kill infected T cells [130]. By increasing the expression of dendritic cell-specific ICAM-grabbing non-integrin on DCs, which traps infectious HIV particles, Nef favours spreading of HIV to T cells [131]. Furthermore, differently from Tat, Nef induces Fas-L upregulation and apoptosis in bystander cells both *in vitro* and *in vivo* [132,133]. Finally, infection of monocyte-derived dendritic cells (MDDCs) with a full-length, 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 [134].

Rev is also absolutely required for HIV replication as it facilitates the nuclear export of intron-containing viral mRNAs allowing the transition from the early to late phase of gene expression, and proviruses lacking Rev do not produce virions [135]. In contrast to Tat and Nef, whether Rev is released at cellular level and exerts effects on neighbouring cells is presently unknown. However, like Tat and Nef, Rev is often targeted by CTLs in HIV-positive individuals [90] and is broadly conserved among different HIV-1 clades in its functionally constrained and immunodominant domains at the N terminus. However, spontaneously occurring mutations in Rev have been reported to reduce HIV-1 structural gene expression to levels undetectable by CTLs, and may represent a mechanism to escape immune recognition [136].

Taken together, these data suggest that HIV-1 Rev, Tat and Nef play important roles in the pathogenesis of HIV/AIDS disease by facilitating transmission across the mucosal barrier, viral replication and dissemination, while impairing immune responses. Nevertheless, besides their pathogenetic effects, which constitute a solid substrate for their inclusion in
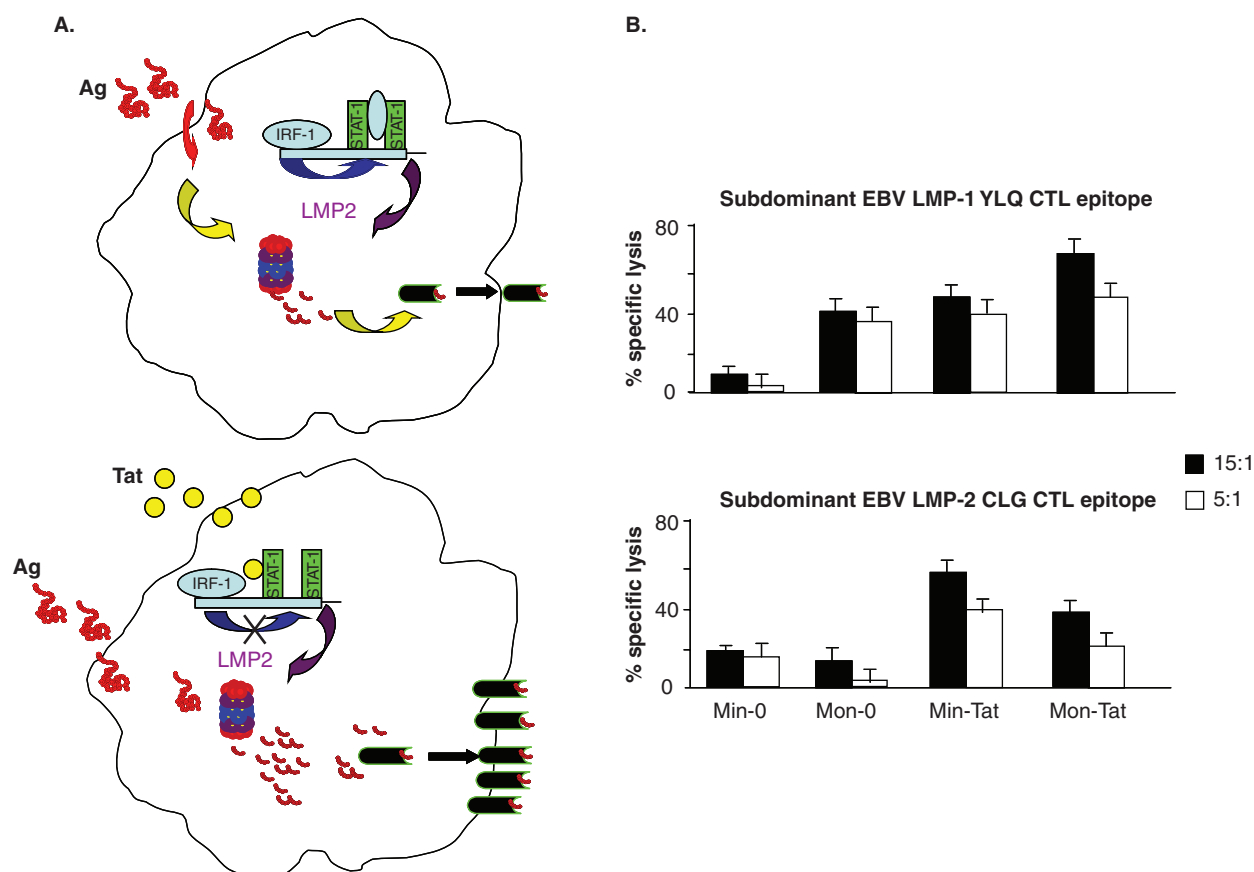
Figure 3. Schematic representation of the effect of Tat on the immunoproteasome. A. Tat increases the processability of the antigens allowing the generation of epitopes, otherwise cryptic, to be presented by the antigen-presenting cell. **B.** EBV-transformed human B cells (Min cells: HLA-A2+ and HLA-A11+; and Mon cells HLA-A2+ and HLA-A11-) transduced with pBabeP (Min 0; Mon 0) or with pBabeP-Tat (Min-Tat; Mon-Tat) expressing HIV-1-Tat, were used as a target in cytotoxicity assays of CTLs specific for the HLA-A11 and HLA-A2 presented EBV subdominant CTL epitopes.

Ag: Antigen; CTL: Cytotoxic T lymphocyte; EBV: Epstein–Barr virus; HLA: Human leukocyte antigen; IRF: IFN regulatory factor; LMP: Latent membrane protein; MDDC: Monocyte-derived dendritic cell; STAT: Signal transducer and activator of transcription.

vaccine formulation, Tat and Nef (nothing is known for Rev) also display immunomodulatory features that make them attractive adjuvants for other antigens.

MDDCs efficiently and rapidly take up native, but not oxidised-inactive, Tat (**Figure 2**) by receptor-mediated endocytosis. Native Tat promotes MDDC maturation and activation (increased expression of MHC antigens and costimulatory molecules) and production of T_H1 cytokines and chemokines (IL-12p40, TNF-α, macrophage inflammatory protein [MIP]-1α, MIP-1β, RANTES), leading to both a more efficient presentation of both allogeneic and exogenous soluble antigens and T_H1 polarisation [137]. In addition, Tat modifies the catalytic subunit composition of the immunoproteasome in B and T cells either expressing Tat or treated with exogenous biologically active Tat protein [138,139]. These changes correlate with an increase of the major proteolytic activities of the proteasome and result in a more efficient generation and

presentation of subdominant MHC class l-binding CTL epitopes of heterologous antigens (**Figure 3**) [139]. Thus, the modifications of antigen processing and generation of CTL epitopes by Tat may have an impact both on the immune-mediated control of infected cells during HIV-l infection and vaccination strategies. In fact, co-administration of HIV-1 Tat and Ad-SIV-gag induced a significant enhancement of Gag-specific cellular responses (T_H1 and CTL) in immunised mice [140].

On the other hand, HIV-1 recombinant Nef protein enters uninfected human monocytes and induces activation of bystander T cells, upregulating IL-15 production. On immature DCs Nef upregulates the expression of surface molecules (CD1a, HLA-DR, CD40, CD83, CXCR4 and to a lower extent CD80 and CD86), and stimulates the production of cytokines (IL-1, IL-12, IL-15, TNF-α) and chemokines (MIP-1α, MIP-1β, IL-8), thus promoting DC differentiation toward fully competent APC [141]. In contrast, other studies

have shown that Nef delivered to MDDCs by viral vectors downregulates CD4 expression, but has little or no effect on other surface molecules, including MHC class I molecules as well as MDDC maturation [142]. Although these contrasting results may depend on the different systems used (recombinant protein versus vectored protein expression), they support an immunomodulating activity of Nef. In this regard, upregulation of IL-15 was observed in monkeys vaccinated with live-attenuated SIV ΔNef virus and found to correlate with protection following a subsequent challenge with SIV [143].

Thus, several lines of evidence suggest the usefulness of a non-structural protein-based vaccine and represents the rationale of choosing this approach for an HIV/AIDS vaccine candidate:

- as mentioned above, Tat, Rev and Nef are expressed very early after infection and strongly dysregulate the immune system contributing importantly to the establishment of the infection
- Rev, Tat and Nef are remarkably conserved in their immunogenic regions (both B- and T-cell epitopes)
- Tat and Nef display immunomodulatory effects on APCs exerting an adjuvant effects and possibly driving the type of adaptive immune responses
- in asymptomatic individuals, responses to these non-structural proteins significantly correlated with the non-progression to disease
- Tat, Rev and Nef either alone or in combination have been shown in preclinical models to be safe, to elicit broad and specific immune responses and, more importantly, to control viral replication and to block disease progression

In seropositive patients, vaccination with non-structural proteins may contribute to reducing HIV-1 replication and disease progression. In individuals exposed to the virus after vaccination, the vaccine could modify the virus–host dynamics at the very beginning of the infection, lessening or halting clinical progression.

Based on these results and considerations, Phase I trials have been conducted with Tat or Nef. In particular, preventative and therapeutic trials have been conducted in Italy with the Tat protein and the results indicate that the Tat vaccine is safe and immunogenic both in the preventative and in the therapeutic settings and will proceed to Phase II trials [144]. Similarly, vaccination with a modified vaccinia Ankara vector expressing Nef was safe and able to stimulate anti-HIV immunity in chronically HIV-1-infected volunteers [145].

5.3 Mucosal immunity

Targeting DCs or immune cells at the mucosal barrier might not be enough to induce efficient mucosal and systemic immune responses. To overcome this obstacle, efforts are directed to: i) find efficient means to deliver vaccine antigens, particularly when a native form is used; and ii) develop safe adjuvants or immunoadjuvants that will increase the potency of the immune responses generated by the vaccine antigens. Among the numerous adjuvants, cholera toxin or the closely related heat labile enterotoxin are among the most potent mucosal adjuvants. However, they are toxic for human use and, therefore, truncated or derivative forms have been developed and tested in preclinical model [146,147]. Similarly, a number of other mucosal adjuvants have been developed including macrophage-activating lipopeptide 2 [148] and immunostimulating complexes [149]. Of note, ginseng and Salviae have been reported as mucosal adjuvants against influenza virus as well as immunomodulators during influenza virus infection [150].

The combination of alternative molecules such as cytokines, chemokines and CpG-rich oligodeoxynucleotide in a vaccine approach is an expanding research field. Bacterial DNA or synthetic ODNs containing unmethylated CpG motifs have the ability to mobilise potent immune responses in mucosal tissues. Their immunomodulatory effects are improved when chemically conjugated with the non-toxic B subunit of cholera toxin [151]. Furthermore, cytokines and chemokines have been reported to synergise with other adjuvants in enhancing mucosal immunity [152-155]. In addition, adjuvants may also affect the quality of immune response, including T_H1 or -2 polarisation. In this respect, it was shown that a detoxified adenylyl cyclase (CyaA) from *Bordetella pertussis* carrying HIV-1 Tat targets DCs and, in contrast to Alum, induces T_H1 Tat-specific T-cell responses. The CyaA–Tat vaccine was also able to generate anti-Tat neutralising antibodies in monkeys [156].

Thus, a number of molecules may act synergistically to improve mucosal immunity. However, at least two points should be considered. First, extensive testing in preclinical models is required in order to select the best chemical or biological adjuvant for human use. Second, a better understanding of how Rev, Tat or Nef affect viral infectivity and tropism, modulate HIV trafficking across mucosal surfaces, drive recruitment of target cells and induce immunodysregulation at the port of entry, and how these events relate to progression to AIDS, is crucial for the rational development of both preventative and therapeutic approaches against HIV/AIDS, including the selection of the proper adjuvant.

An additional challenge in the HIV/AIDS vaccine field is the development of a safe system to deliver vaccine antigens, which is easy to handle without cold chain infrastructures, and maintains intact the biological and immunogenic properties of the antigen, while targeting key elements at mucosal inductive sites. Use of attenuated recombinant vectors or biologically inert materials for priming potent, persistent immune responses and targeting mucosal inductive sites are considered new vaccine strategies.

Among the numerous and promising live-attenuated vectors (measles virus, herpes virus, Sendai virus, Venezuelan equine encephalitis virus, attenuated *Salmonella*, modified-vaccinia virus, adeno-associated virus) [157-159], replicating adenoviruses (Ad) are attractive and highly promising, as they
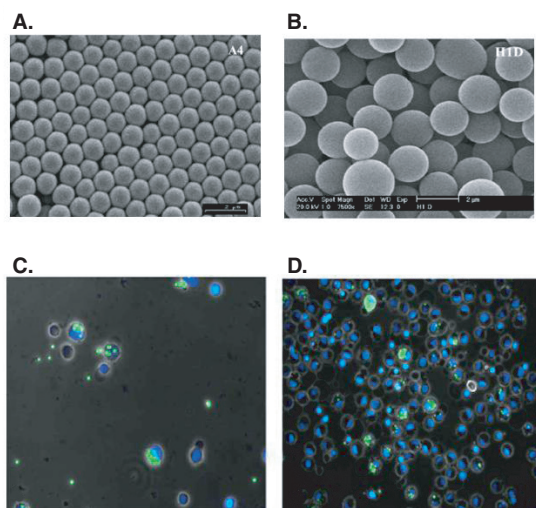
Figure 4. Polymeric microparticles and their uptake by monocytes and DCs. SEM images of polymeric anionic microparticles made of **A.** poly(styrene) and poly(vinyl alcohol) stabiliser (A4 microparticles), and **B.** poly(methyl methacrylate) and Eudragit L100/55 stabiliser (H1D microparticles). Human monocytes **C.** and MDDCs **D.** were cultured in the presence of fluorescent (H1D) microparticles for 24 h, fixed with paraformaldheyde and observed at fluorescent and confocal microscopes. Reprinted from CAPUTO A, BROCCA-COFANO E, CASTALDELLO A *et al.*: Novel biocompatible anionic polymeric microspheres for the delivery of the HIV-1 Tat protein for vaccine application. *Vaccine* **22**(21-22):2910-2924, Copyright (2004), with permission from Elsevier [164].
DC: Dendritic cell; MDDC: Monocyte-derived dendritic cell;
SEM: Scanning electron microscopy.

infect the epithelial cells lining mucosal surfaces. Of note, replication-competent Ad recombinants at the same or lower dose as replication-defective vectors have been shown to be more immunogenic that the replication-defective counterpart, to elicit both systemic and mucosal cellular immune responses, and to prime broad antibody responses [112]. Replicating Ad–SIV recombinants in combination with Env subunit boosting have elicited potent, durable protection against SIV$_{mac251}$ challenge [160,161]. Thus, mucosal administration even in the lyophilised form of these vectors and in particular replicating Ad (Robert-Guroff M, unpublished results), may represent an important component of a future combination strategy incorporating both structural and non-structural vaccine antigens.

Non-reactogenic substances such as microparticles are also thought to be an important tool to deliver vaccine antigens, because they are devoid of the toxic effects shown by other adjuvants and do not elicit antivector responses observed in the case of antigens delivered by live-attenuated vectors. As recent studies have indicated that the HIV-1 Tat protein represents a promising candidate of a prophylactic

and/or therapeutic vaccine against AIDS and as Tat contains a positively charged domain, rich in arginine and lysine [162], it was chosen as a model antigen to be delivered by microspheres. Novel anionic microspheres were developed and evaluated for their capability of reversibly adsorbing a biologically active HIV-1 Tat protein, preserving its native form and shelf-life and efficiently delivering it *in vitro* or *in vivo* in the absence of toxic effects (**Figure 4**). Furthermore, safety and immunogenicity studies in mice and monkeys indicate that these microparticles represent a promising model system to deliver protein-based vaccines in which the preservation of the native conformation and biological activities of the antigen are essential (Ensoli B, unpublished results) [107,163,164].

5.4 Tat, Nef, Rev and modified Env combination approaches

Epidemiological and experimental evidence support the development and evaluation in Phase I trials of an entirely new generation of vaccines based on the rational combination of non-structural (Rev, Tat, Nef) and structural (ΔV2-Env) HIV-1 gene products for induction of effective, long-lasting and possibly sterilising antiviral immunity. Indeed, by combining the immunogenic and immuno-modulatory properties of non-structural proteins with the ability of modified Env to generate broad and cross-protective neutralising antibodies, sterilising immunity, which is the primary goal of a vaccine, may be achieved. However, due to potential antigen interference and immunodominant effects of Env, the new vaccine design, formulation and immunisation protocol must be evaluated and optimised before proceeding to human trials. This is being carried out within the AIDS Vaccine Integrated Project (AVIP) (EU funded Integrated Project [404]), the Mucosal Vaccines for Poverty Related Diseases (MUVAPRED), and the Italy–USA cooperation (scientific network between the Italian and American National Institutes of Health) aimed at developing an effective HIV/AIDS vaccine.

6. Competitive environment

Since the late 1990s, as a consequence of the critical mass of data generated to elucidate the functions of the non-structural genes of HIV, new vaccine approaches have been developed and tested first in non-human primate models and then in clinical trials. These include vaccine combinations containing non-structural genes (Rev, Tat, Nef) either alone or associated with Env or other structural genes (**Tables 2** and **4**). In particular, novel combined vaccine strategies are under evaluation within the European-funded AVIP. The different strategies involve a 'minimalistic' approach in which only two antigens, a regulatory (Tat or Nef) and a structural (ΔV2-Env) HIV protein, are combined, and a 'maximalistic' approach that imitates a live-attenuated vaccine, and, therefore, combines many HIV structural and non-structural genes [165-168].

7. Potential development issues

Advanced clinical trials (Phase II/III) must be performed in developing countries in which the incidence of HIV infection remains high and the need for an effective vaccine is most pressing. Selection of appropriate populations for Phase II/III studies relies on the availability of epidemiological and immunological data as well as strong logistics.

Preparatory studies before conducting Phase II/III vaccine trials are, therefore, needed and must include: i) studies on the incidence and prevalence in the regions targeted for vaccination; ii) investigation of the background characteristics of the immune response to HIV infection both in healthy and in infected individuals; iii) evaluation of the immune cross-recognition of candidate vaccine antigens; iv) building of clinic and laboratory capacity, technology transfer and training; and v) community involvement and harmonisation of different international and local institutions. Another important challenge in conducting HIV vaccine trials in developing countries is to establish a recruitment strategy in order to identify, recruit and maintain in the study volunteers from the community and guarantee their follow up over a reasonable period of time.

8. Expert opinion and conclusion

The inexorable spreading of the HIV pandemic and the increasing deaths from AIDS in developing countries underscore the urgency for an effective vaccine against AIDS.

So far, the promising results obtained in the preclinical testing of Tat, Nef or ΔV2-Env as single vaccine candidates suggest that the combination of non-structural and structural proteins (Tat/ΔV2-Env or Nef/ΔV2-Env or Tat/Nef/ΔV2-Env) may lead to a vaccine formulation with superior efficacy having a chance to fulfill the primary goal of the HIV/AIDS global vaccine strategy. Nevertheless, new efforts should be made to identify novel Env epitopes and to incorporate them in a potential vaccine strategy useful for both preventative and therapeutic interventions. The next decade will be critical in determining the rate at which a vaccine candidate with acceptable efficacy in preclinical studies and proven safety in Phase I studies will move into Phase II/III clinical trials.

Oral delivery of lyophilised, replication-competent Ad recombinants or non-reactogenic microparticles will facilitate vaccine administration, while also priming potent, persistent immune responses both systemically and at mucosal sites. In addition, non-reactogenic delivery systems and inclusion of immunomodulating factors in the vaccination approach may contribute to: i) solving pratical aspects of vaccination (stability of the antigen, no cold chain constraints, ease of transportation and administration); and ii) increasing the immunogenicity of the vaccine without adverse effects.

To meet this unprecedented challenge, a close synergy between highly qualified scientists from the public and private sectors is critical to maximise chances of success. In this respect, the constitution of consortia (such as the European Consortia for HIV vaccine development, including the AVIP, the MUVAPRED, the Very Innovative AIDS Vaccine, and the Neutralising Antibody Consortia and Live Attenuated Virus Consortia) and, more recently, of the 'global HIV vaccine enterprise' [169] represents a major advancement. They may provide the HIV vaccine field with the critical mass necessary to speed up vaccine development and testing. In line with this, because the HIV epidemic is a global public health problem, governments are expanding strategic investments in HIV programmes and technologies aimed at facilitating national and international partnership(s) for development of joint programmes.

Acknowledgements

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