

Forum in immunology

Vaccines based on the native HIV Tat protein and on the combination of Tat and the structural HIV protein variant Δ V2 Env

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Abstract

The promising results obtained with the HIV-1 Tat-based vaccines in mice, monkeys and humans, a better understanding of Tat immunomodulatory functions, as well as evidence that vaccination with trimeric V2 loop-deleted HIV-1 Env induces cross-clade neutralizing antibodies led to the rational design of a novel vaccine based on the combination of Tat and V2-deleted Env.

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1. Introduction

HIV-1 Tat-based vaccines (both DNA and protein) have proven to be safe and immunogenic in preclinical models, and effective in controlling virus replication and blocking disease onset in monkeys [1–4]. In addition, recent results indicate that incidence and risk of progression to advanced HIV disease is lower among individuals with antibodies (Ab) against Tat as compared to anti-Tat negative individuals [5], and that clade-B Tat is effectively recognized by sera from individuals infected by different virus clades (A,B,C,D) in Africa [6], thereby supporting the concept of a cross-clade vaccine. These data indicate that Tat can be an optimal target for vaccine strategies in populations where different HIV-1 sub-types are prevalent. Recently, randomized, double

blinded and placebo controlled phase I preventive and therapeutic trials to evaluate primary (safety) and secondary (immunogenicity) endpoints upon vaccination with the native Tat protein have been completed in Italy.

In addition to its antigenic properties, Tat displays immunomodulatory features, which makes it an attractive adjuvant for other antigens and which can be exploited for vaccination strategies aimed at driving or increasing Th-1 type immune responses and cytotoxic T lymphocyte (CTL) activities against HIV structural antigens.

With regard to Env, since it is responsible for virus binding and entry into cells, the rationale for developing an Env-based vaccine is to generate neutralizing Ab capable of protecting from infection (sterilizing immunity). Given the failure of the first phase III clinical trial based on monomeric wild type Env, novel modifications have been introduced into the Env protein with the aim to provide better exposure of cryptic neutralization-sensitive epitopes and to obtain a reliable immunogen for broadly reactive Ab generation. In particular, investigators of the AIDS Vaccine Integrated Project (AVIP) Consortium have produced for clinical evaluations novel HIV-1 trimeric Env immunogens from sub-types B and C, which are deleted in the V2 region of the protein [V2-deleted Env (Δ V2 Env)]. Such immunogens have been proven able to elicit broader immune responses than their wild-type counterpart [7].

Abbreviations: Ab, antibodies; AVIP, AIDS Vaccine Integrated Project; CTL, cytotoxic T lymphocyte; Δ V2 Env, V2-deleted Env; ELISA, enzyme-linked immunosorbent assay; GMP, good manufacturing practice; i.d., intradermal; IFN γ , interferon γ ; IL, interleukin; IVAP, in vitro antibody production; MID, monkey infectious dose; PBMC, peripheral blood mononuclear cells; PCR, polymerase-chain reaction; PHA, phytohemagglutinin; SHIV, simian-human immunodeficiency virus; SIV, simian immunodeficiency virus; SOPs, standard operating procedures; TNF α , tumor necrosis factor α .

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Therefore, epidemiological and experimental evidence exists that constitutes the basis to develop and evaluate in phase I trials a new generation of vaccines based on the rational combination of regulatory (Tat) and structural (Δ V2 Env) HIV-1 gene products to support an effective, long-lasting and possibly even sterilizing antiviral immunity. However, due to potential antigen interference and immunodominant effects of Env, the new vaccine design, formulation and immunization protocols must be evaluated and optimized before proceeding to clinical development.

2. The Tat vaccine—monkey safety and immunogenicity studies

HIV-1 Tat-based vaccines (both protein and DNA) have proven to be safe and immunogenic in mice and protective in monkeys [1–4]. To gain more insights into the Tat protein vaccine, which is a key component in several of the combined vaccine candidates that are part of the AVIP program, a brief discussion of former preclinical data is provided. Seven adult male cynomolgus monkeys (*Macaca fascicularis*) were vaccinated with a native biologically active HIV-1 Tat protein (Table 1).

Specifically, six monkeys were immunized subcutaneously (s.c.) with 10 μ g of Tat and the adjuvant RIBI (three monkeys) or aluminum phosphate (Alum; three monkeys), and one animal with Tat (6 μ g), intradermally (i.d.), in the absence of adjuvants. Additional injections were given at 2, 6, 11, 15, 21, 28, 32 weeks after the first immunization, and the last boost was given intramuscularly at week 36 with Tat in immune-stimulating complexes, except for the monkey vaccinated with Tat i.d., which did not receive this last boost. Two monkeys served as controls and were injected with RIBI or Alum alone, respectively (Table 1).

No toxicity (acute or chronic; local or systemic) was ever detected in the vaccinated animals throughout the immunization period as assessed by monitoring of vital signs including body weight, hydration and rectal temperature, examination

of integument, respiratory, cardiovascular, gastrointestinal, musculoskeletal and lymphatic systems; evaluation of hematological, immunological and biochemical parameters (data not shown). Of note, at each physical examination all inoculation sites were carefully searched for signs of local reaction.

The six monkeys inoculated with Tat and RIBI or Alum developed high titers of specific Ab, whereas anti-Tat Ab titers were low and transient in the animal vaccinated i.d. In addition, plasma from the monkeys inoculated with Tat and RIBI or Alum monkeys were capable of neutralizing the activity of increasing concentrations of exogenous Tat protein (120–500 ng/ml), as compared with the pre-immune plasma, in a test of rescue of Tat-defective proviruses induced by the addition of extracellular Tat and measured by determining p24 antigen production in the cell supernatants [8–10].

Tat vaccination also induced specific cellular immune responses. Specifically, all the monkeys vaccinated with Tat and RIBI or Alum showed a Tat-specific proliferation, whereas no response was detected in the macaque vaccinated i.d. with Tat alone and in the control animals [1]. In the vaccinated monkeys anti-Tat CTL were detected at week 36 after the first immunization. These data were confirmed by measuring the production of tumor necrosis factor (TNF) α , a known mediator of CTL activity [11–14], after stimulation of fractionated peripheral blood mononuclear cells (PBMCs) with Tat. Only the animals that showed an anti-Tat CTL activity also produced TNF α upon Tat stimulation, whereas monkeys that were negative for CTL activity produced TNF α upon phytohemagglutinin (PHA) but not Tat stimulation. In addition, cell sorting studies indicated that the main source of TNF α (about 90%) was the CD8+ T cell fraction, confirming the presence of CD8+ anti-Tat CTLs [1].

These results were reproduced in subsequent protocols for both Tat and Alum inoculated s.c. and Tat alone given i.d. (unpublished data). At week 50 after the first immunization (14–18 weeks after the last boost), all animals were challenged intravenously with SHIV89.6P243, a highly pathogenic simian-human immunodeficiency virus (SHIV) con-

Table 1
Tat protein vaccine protocol (modified from [1])

Monkey	Immunogen	Adjuvant	Administration
54844			
54879	Tat protein (10 μ g/250 μ l)	RIBI (250 μ l)	Subcute, 500 μ l in one site (dorsal area, close to the neck)
54963			
54899			
55396	Tat protein (10 μ g/250 μ l)	Alum (250 μ l)	Subcute, 500 μ l in one site (dorsal area, close to the neck)
54240			
54222	Tat protein (6 μ g/ μ l)	Nil	Intradermic, 150 μ l in two sites (upper dorsal area)
55123	Saline buffer (250 μ l)	RIBI (250 μ l)	Subcute, 500 μ l in one site (dorsal area, close to the neck)
55129	Saline buffer (250 μ l)	Alum (250 μ l)	Subcute, 500 μ l in one site (dorsal area, close to the neck)
12	Nil	Nil	
2	Nil	Nil	

Table 2

Tat protein vaccine: immunological responses and fate post-challenge (SHIV-89.6P) of vaccinated and control monkeys

Monkey	Group	Challenge (MID ₅₀)	Virological response				Proliferative response ^e		IFN- γ ELISpot ^f		Anti-SIV Ab ^g	Anti-HIV Env Ab ^g	Anti-HIV Tat Ab ^g
			Plasma viremia ^a	DNA PCR ^b	E-DNA ^c	CD4 ⁺ decline ^d	Tat protein	Gag protein/peptide pool	Tat protein	Gag protein			
54844	RIBI + Tat protein (10 μ g, s.c.)	10	–	+	–	–	++	–	+	–	+/-	+**	++
54879		10	–	+	–	–	+++	++	+	–	+/-	+**	++
54963		10	+	+	+	+	+++	+	+	+	+	+	++
54899	Alum + Tat protein (10 μ g, s.c.)	10	–	+	+	–	+++	+	+	–	+/-	+**	+++
55396		10	+	+	+	+	+	++	+	+	+	+	+++
55240		10	–	+	–	–	++	+	+	–	+/-	+**	+++
54222	Tat protein (6 μ g, i.d.)	10	–	+	–	–	+++	+	+	+	+/-	+**	±
55123	Control RIBI	10	+	+	+	+	++	+++	–	+	+	+	–
55129	Control Alum	10	+	+	+	+	++	+++	–	+	+	+	–
12	Nil	2.8	+	+	+	+	ND	ND	–	–	+	+	ND
2†	Nil	28	+	+	ND	+	ND	ND	ND	ND	–	+	ND

* Proviral DNA was detected sporadically (<5 times) and at very low copy number (1–10 copies per μ g DNA). ** Anti-HIV Ab detected by IVAP in the supernatants of PBMCs stimulated with pokeweed mitogen. † Monkey 2 was euthanized at week 35.

^a Until week 14 post-challenge quantitation of SHIV89.6P RNA copies was performed only in the Bayer-Chiron Diagnostics Reference Testing Laboratory (Amsterdam, The Netherlands) by a branched-DNA (bdNA) signal amplification assay recognizing the pol region of the SIVmac251 strain, as described in [1], with a cut-off of 1500 RNA copies per ml. Between weeks 14 and 28 post-challenge quantitation of SHIV89.6P RNA copies were performed by both the bdNA method and by a more sensitive quantitative-competitive RNA-polymerase chain reaction (QT-RNA-PCR, cut-off: 50 RNA copies per ml) as already described in [15]. Starting from since week 35 post-challenge quantitation of SHIV89.6P RNA copies was performed only by QT-RNA-PCR. Results above or below the cut-off values were expressed as positive (+) or negative (–), respectively.

^b DNA was extracted from whole blood or from lymph node cells. SHIV proviral copy number was determined by a semi-quantitative DNA PCR utilizing 1 μ g of DNA and amplifying a 496 bp region of the *gag* gene of SIVmac239, as already described in [1]. Assay sensitivity was one SHIV proviral copy per μ g of DNA; + indicates proviral copy numbers > 1.

^c Viral extrachromosomal circular DNA (E-DNA) was extracted from lymphocytes (2–5 \times 10⁶) and analyzed by PCR for the presence of circular forms of viral DNA as described in [16,17].

^d Citrated peripheral blood cells were stained with phycoerythrin PE-conjugated anti-CD4 (Biosource International, Camarillo, CA) and peridin chlorophyll protein (PerCP)-conjugated anti-CD8 mAb (Becton-Dickinson, Mountain View, CA), and analyzed with a FACScan cytometer and software (Becton-Dickinson) as described in [1]. Absolute cell numbers were calculated from blood cell counts. Depletion of CD4+ cells was evaluated by the decrease in the absolute number of CD4 + T cells > 50%.

^e Stimulation index \geq 3 was considered positive. Peak of stimulation index (ratio between Tat- and Gag-specific and the control proliferative response): –, < 3; +, 3–10; ++, 11–30; +++, > 30.

^f IFN γ production was measured by a commercial kit (Human IFN γ ELISpot, Euroclone, Paignton, UK), following the manufacturer's instructions. Only tests in which the number of spots in response to PHA and anti-CD3 mAb was > 100 and > 50, respectively, were considered. A test was considered positive (+) when the number of spots in the sample was > threefold over that detected in the negative control. Cut-off was established on data obtained with PBMCs from 24 naïve animals. Monkey 2 could not be analyzed since it was euthanized at week 35 post-challenge.

^g Ab titers to the whole SIV were determined by endpoint dilution of plasma samples using a HIV-2 ELISA (Elavia AC-Ab-Ak II Kit, Diagnostic, Pasteur) according to the manufacturer's instructions. Ab titers against the HIV-1 Env were detected by an HIV-1 ELISA assay (HIV-1/HIV-2 Third Generation Plus, Abbott, Chicago, IL). The mean of the negative control plus 3 S.D. represented the cut-off value. IVAP upon pokeweed mitogen stimulation of PBMCs was performed as described earlier in [1]. Anti-Tat Ab titers were determined by ELISA (see legend to Table 4). Results of anti-SIV, anti-HIV and anti-Tat Ab production above, equal or below the cut-off value were expressed as positive (+), border line (+/-) or negative (–), respectively.

taining the tat gene of HIV-1. To determine titer and virus pathogenicity of this virus in cynomolgus monkeys, the original virus SHIV89.6P (grow in rhesus monkeys) and the derived SHIV89.6P243 virus stock obtained upon passage in cynomolgus macaque 243, were inoculated into six and eight monkeys, respectively. High levels of viral replication (including p27 antigenemia, plasma viremia, proviral DNA copies and cytoviremia), a profound and persistent decrease in CD4 T cell counts and a high rate (> 60%) of animal death were observed in the inoculated monkeys independently from the virus stock utilized, and no differences were found when the data obtained from these animals were compared with those

already published [2]. Therefore, all vaccinated and control macaques were challenged intravenously with 10 monkey infectious doses (MID₅₀) of SHIV89.6P243. As an additional control of the virus inoculum, two naïve animals (monkeys 2 and 12), were inoculated with a threefold higher (28 MID₅₀) or lower (2.8 MID₅₀) viral inoculum, respectively (Table 2).

After challenge, all the control monkeys, including the naïve animals, and the two monkeys injected with RIBI or Alum alone were overtly infected, as indicated by the high levels of p27 antigenemia [detected by enzyme-linked immunosorbent assay (ELISA)] and plasma viremia [determined

by both branched-DNA and quantitative-competitive RNA-polymerase-chain reaction (PCR)] (Table 2). In contrast, only two out of the seven Tat protein-vaccinated monkeys (one given Tat and RIBI and one Tat and Alum) were overtly infected as shown by both these assays. For all the other five vaccinees p27 and viral RNA were always undetectable in plasma for up to 104 weeks post-challenge (Table 5 and Ref. [4]). Further, simian immunodeficiency virus (SIV) proviral DNA was sporadically detected at very low copy number in all the protected monkeys. In contrast, PBMCs from the controls and the two vaccinated and infected animals had a very high proviral copy number since week 2 after challenge that remained always detectable (Table 2). Virus isolation carried out with CD8-depleted PBMCs stimulated with PHA and recombinant human interleukin 2 (IL-2) (rhIL-2), or with anti-CD3 Ab and rhIL-2, resulted always negative in the five protected macaques vaccinated with Tat. Conversely, virus was always isolated and cell-associated viremia (cytoviremia) was high in the four control animals and in the two vaccinated and infected monkeys.

The highest anti-SIV Ab titers were detected in the control monkeys, followed by the two infected and vaccinated animals in which appearance of Ab was delayed and titers were at least 2 logs lower as compared to the control macaques. Low anti-SIV Ab titres (1:2 to 1:100) were detected in the five protected monkeys and they became negative over time. Anti-HIV-1 Env Ab were detected only in sera from the infected animals. However, they were detected at week 14 after challenge by *in vitro* Ab production (IVAP) in the supernatants of PBMCs from all the monkeys upon stimulation with pokeweed mitogen (Table 2, and Ref. [1]).

The number of CD4+ T cells was consistent with the data of the virological assays. Specifically, in all protected monkeys the CD4 T cells remained in the normal range after the viral challenge and during all the follow-up period, whereas they decreased considerably in all the controls and in the two vaccinated and infected macaques (Table 2). In fact, protection was durable, since no signs of virus replication were found neither in PBMC nor in lymph nodes of the protected animals (five out of seven vaccinated macaques) during the 2 years of follow-up [4]. Further, no residual virus hidden in resting cells was detected in any of the protected macaques, either in the plasma or in lymph nodes, upon two boosts (at weeks 65 and 68 after the challenge) with tetanus toxoid (40 IU per dose, intramuscularly, of Anateal), a stimulus known to activate virus replication. In contrast, virus persisted and replicated in PBMC and lymph nodes of infected animals, two of which died.

Protection correlated (100%) with the presence, prior to challenge, of anti-Tat specific CTLs and with TNF α production by CD8⁺ T cells upon Tat stimulation. However, this does not exclude that anti-Tat Ab play a role in protection, since the two vaccinated and infected monkeys, which did not have CTLs nor TNF α production at pre-challenge time but had high titres of anti-Tat Ab, showed lower levels (and delayed appearance) of anti-SIV Ab as compared to the controls, sug-

gesting that they were exposed to a lower antigen load. Long-term protection correlated with the presence of high and stable humoral and cellular (CD4 and CD8 T cells) responses against Tat [4]. In contrast, anti-Gag humoral and cellular (CD4 and CD8 T cells) responses were consistently and persistently positive only in the monkeys that did not control primary virus replication. Finally, a pilot study conducted in SHIV infected macaques indicated that vaccination with either Tat DNA or protein is safe also in monkeys with AIDS, as indicated by plasma viral loads and CD4 T cell counts whose values and kinetics remained undistinguishable from those detected in the control group (unpublished data).

3. Tat vaccine manufacturing, approval for human use and phase I trial conduction

The vaccine development and approval process is a systematic approach that ensures that a vaccine receives thorough testing and scrutiny before being administered to humans. This process requires the accomplishment of several tasks. The candidate vaccine has to undergo preclinical assessment and, after safety and immunogenicity results are confirmed in preclinical models, the selected vaccine candidate has to be produced in accordance with the principle and the detailed guidelines of good manufacturing practice (GMP) for medicinal products. The application of GMP to the manufacture of investigational medicinal products is intended to ensure that trial subjects are not placed at risk, and that the results of clinical trials are unaffected by inadequate safety, quality and efficacy arising from unsatisfactory manufacture.

3.1. Tat vaccine manufacturing

The active substance of the anti-HIV-1/AIDS Tat-based vaccine is represented by the biologically active recombinant HIV-1 Tat protein (HTLV-III_B strain, clone BH-10). The Tat protein has been produced under GMP conditions in order to be administered in humans. The process of production has been implemented to allow a scaling-up in a GMP setting, guaranteeing that no modifications will result from this process with respect its specifications (physicochemical properties, immunochemical properties, biological activity), to provide the necessary amount of the active substance and to comply with the acceptance criteria for product- and process-related substances.

Specifications have been chosen to confirm the quality of the investigational medicinal product and are focused on those molecular and biological characteristics found to be relevant to ensure the efficacy of the product, represented in this particular case by the biological activity and monomeric form of the Tat protein. A list of tests (Table 3) have been selected according to a set of criteria to which the recombinant Tat protein should conform, to be considered acceptable for its intended use, such as vaccination. In addition, in-process controls have been defined to guarantee the reproducibility of

Table 3
Specification and testing for the physicochemical, immunochemical and biological characterization of Tat protein

Specifications	Tests
Physicochemical properties	- Molecular weight (SDS-PAGE, Comassie blue staining, silver staining) - HPLC
Immunochemical properties	- Western blot
Biological activity	- Uptake by dendritic cells

the process and specific tests will have been performed to verify the absence of new as well as already known impurities and contaminants in the final clinical preparation.

The recombinant Tat protein was obtained from a lysate of engineered *E. coli* cells. The pET3c-Tat and pLysS plasmids were constructed and optimized for Tat expression, and transferred in an *E. coli* strain, selected for optimal growth and protein expression. The lysates obtained from the engineered *E. coli* cells were filtered and purified by DEAE chromatography followed by heparin sepharose chromatography. These processes do not involve the use of toxic chemicals, allowing the purification of the desired product in safe conditions. No bovine or animal materials have been used in the process of production of the GMP Tat vaccine. The purified Tat protein has been then formulated in potassium phosphate saline buffer containing sucrose and human serum albumin. This formulation has been defined in order to maintain the biological activity of the protein in a liquid form, when the vaccine is stored at -80°C . After formulation and filling of the vaccine at the required dosages, the vaccine ampoules have been packaged and labeled. Being the phase I clinical trial a randomized, double blind, placebo controlled trial, a system has been put in place to ensure that the blind was achieved and maintained while allowing for identification of “blinded” product when necessary. The storage conditions of the vaccine have been accurately detailed in the clinical protocols and the cold chain, from the manufacturer to the clinical sites, has been strictly monitored. In addition, quality control have been performed by evaluating the biological activity of the vaccine during the clinical trial.

3.2. Approval for human use and phase I clinical trials conduction

The drug approval process is very orderly and well defined by national (Italian) and European guidelines for the evaluation of composition and safety of new active substances. A dossier has been prepared and submitted to the Italian Ministry of Health for the approval for human use of the Tat vaccine. The dossier included the following information:

- Documentation for the assessment of composition (GMP certification, description of the process of production; description of the physico-chemical specification of the active substance);
- Documentation for the assessment of pharmacology (immunogenicity);
- Documentation on toxicology and safety;

• Clinical protocol.

The dossier has been revised by a Committee of International Experts. After the approval for human use has been granted, the required documentation was submitted to the central (Istituto Superiore di Sanità, Rome, Italy) and local Ethics Committees.

Phase I preventive and therapeutic trials in HIV-infected (therapeutic) and non-infected (preventive) individuals have started (November 2003) in Italy in four validated clinical centers [San Raffaele Hospital (Milan), San Gallicano Hospital (Rome), Policlinico Umberto I (Rome), Spallanzani Hospital (Rome)]. The primary endpoint of these vaccine trials was to qualify Tat protein as safe for evaluation in phase II clinical trials. The secondary endpoint was to qualify the Tat protein as immunogenic for evaluation in phase II clinical trials. Both trials were randomized, placebo controlled, and double blinded. For both trials, volunteers have been randomized to one of two treatment arms with different routes of administration (subcutaneous in association with the Alum adjuvant or i.d. without adjuvant) and blinded to dosage group (7.5, 15, 30 μg of Tat protein and placebo). Volunteers are being monitored for anti-Tat specific Ab, interferon γ (IFN γ) and IL-4 production in response to Tat (by ELISpot) and anti-Tat proliferative response in vitro. All volunteers have been enrolled and completed the cycles of vaccination. Data are being elaborated, however the preliminary results indicate full accomplishment of both primary and secondary endpoints.

3.2.1. Harmonization of clinical, laboratory and social-ethical platforms

In order to achieve clear and comparable read-outs from the trials and to guarantee high levels of internal and external quality controls, the clinical evaluations and laboratory procedures, as well as psychosocial and behavioral assessments have been harmonized along common GCP/cGLP procedures. Principal objectives of such harmonization included: a) definition of standard operating procedures (SOPs); b) technology transfer; c) training of personnel among clinical centers and core laboratories.

3.2.1.1. Clinical platform. Harmonization of the clinical platform included the preparation of common clinical protocols to be defined according to the results of preclinical studies. In addition, SOPs have been prepared and implemented to standardize critical steps in the setting of clinical trials. These include: communication and enrollment procedures, pre-screening procedures, randomization procedures, vaccine storage, preparation for injection of the vaccine formulations, vaccine administration, management of adverse events, blinding and unblinding procedures, collection and shipment of biological samples.

3.2.1.2. Laboratory platform. Immunological evaluation apply state-of-the art technologies and methodologies, standardized criteria and procedures according to common SOPs and protocols. The standardized assays include:

- Humoral immunity: titration of specific IgG, IgA and IgM; anti-HIV Ab neutralizing activities; B-cell epitope mapping; induction of mucosal (saliva, urine, vaginal washes) HIV-1-specific IgG and IgA.
- Cell-mediated immunity: antigen-specific T cell responses (IFN γ , IL-2, perforin and IL-4 ELISpot assays); intracellular cytokine staining for IFN γ , IL-2, TNF α , combined with phenotypic analysis, T cell proliferative assays CFSE staining; cytokine release by multiparametric analysis.
- Natural/innate immunity: NK cell activity (measurement of perforin), production of CCR5-binding chemokines and α -defensins (multiparametric analysis).
- Human leucocyte antigens typing: all patients will be typed at baseline for both class I and II determinants by commercial PCR-based methodology in order to evaluate the effects of the host genetic background on the response to the AVIP vaccines.

A first line of testing assesses the strength and breadth of B and T cell responses. In particular: a) B-cell responses evaluated by ELISA assays (IgG, IgM, IgA) standardized and validated for the specific vaccine candidate; b) total CD4+ and CD8+ T cell responses tested by IFN γ , IL-2, IL4 and perforin ELISpot assays utilizing both recombinant antigens and peptide pools and/or matrices. A second line assesses: a) antigen-specific cytokine profile (Th1/Th2) by intracellular cytokine staining combined with phenotypic analysis and determination of cytokine release by multiparametric analysis; b) CD4+ and CD8+ T cell proliferative ability (CFSE staining) combined with phenotypic analysis; c) natural immunity by intracellular staining (measurements of perforin), production of chemokines and α -defensins (multiparametric analysis).

Based on these different technologies, a substantial number of parameters for antigen-specific B and T cells can be monitored: absolute frequencies of lymphocytes, lymphocyte subpopulations and functional activities (Ab production, cytokine secretion, degranulation, proliferation, cytotoxicity).

Within the therapeutic trials in infected individuals, plasmatic viral RNA, proviral DNA from PBMC and HIV sequence variability are also being analyzed at several time-points after immunization and are being related to host genetics.

3.2.1.3. Social-ethical platform. A psychosocial protocol has been prepared to complement prescreening procedures in order to identify volunteers that may need specific psychological support throughout the trial and at critical points during the study, such as enrollment, conclusion of the study and follow-up, and in case of screening failure and adverse events. This protocol is based on validated and standardized psychobehavioral testing and will also contribute to reducing dropouts from the study. A psychosocial team, represented by psychologist/psychiatrist selected at each site, has been involved, in cooperation with clinical and laboratory investigators, in the elaboration of the following documentation/

activities: preparation of psychosocial protocol; selection of test for psychological and sociobehavioral testing; preparation and delivery of the informed consent to the volunteers; preparation of the patient information sheet.

4. The Tat and Δ V2 Env combined vaccine—ongoing and future studies

4.1. Safety and immunogenicity studies in small animal models

Within AVIP, the low-cost murine model is being used for safety and immunogenicity screening of a candidate vaccine based on biologically active Tat protein and oligomeric Δ V2 Env protein, in a cost- and time-efficient manner. Immunization with the combined antigens formulation is being compared to vaccination with the single antigens (Tat, Δ V2 Env). Parallel experiments with Tat \pm wild type Env are being performed to compare the immune responses of Env and Δ V2 Env in the presence or absence of Tat. Preclinical studies with these vaccines will be performed to find out the most effective way of delivery. Different parameters, such as antigen dose, route and site of inoculation, different adjuvants (e.g. Alum, MF59), and schedules of immunization are under evaluation in different mice strains (e.g. Balb/c, C57BL6/J). The overall safety of the vaccines will be assessed by monitoring different parameters including: animal weight, food intake and general behavior during the course of immunization, as well as by histological examination of tissues and organs at sacrifice. With regards to immunogenicity, mice will be analyzed for antigen-specific humoral and cellular immune responses. Antigen-specific serum Ab titers (IgM, IgG), epitope mapping and IgG subclasses (e.g. IgG1 and IgG2a) will be assessed on single mouse sera by standardized ELISA tests. In addition, IgA responses will be evaluated in serum and mucosal secretions by ELISA. T cell responses will be analyzed on fresh and on peptide-stimulated splenocytes by i) antigen-specific T cell proliferation tests, ii) CTL assays (⁵¹Chromium release), iii) ELISpot (IFN γ , IL-2 and IL-4) using peptide-based matrix approaches. To define peptide-specific CD4 and CD8 T cell responses, purified CD4 and CD8 T cells will be assayed by ELISpot (IFN γ , IL-2 and IL-4) after stimulation with the responding peptides identified by the matrix-based approach. Alternatively, intracellular staining assays will be used to identify the lymphocyte subpopulation/s responding to the selected peptides.

In order to investigate the virus-neutralizing activity of vaccine-elicited Ab, vaccine formulations that will induce the best anti-Env responses in mice, as assessed by binding assay (ELISA), will be selected to immunize other small animal species (i.e. rabbits) to circumvent the well-known non-specific antiviral activity of mouse serum. In addition, rabbits will allow a further characterization of Ab responses, such as the assessment of Ab avidity and epitope mapping. The breadth of neutralizing activity will be determined by testing

the sera against panels of pseudotyped viruses that represent strains of different clades (A, B, C, E).

Based on the safety and immunogenicity profile in mice, the best vaccine formulations and optimized schedules of immunization will be selected to undergo further safety and immunogenicity studies in monkeys before proceeding to clinical development and to efficacy studies in monkeys.

4.2. Safety, immunogenicity, and efficacy studies in non-human primates

Non-human primates represent a unique and valuable model to evaluate safety, immunogenicity and efficacy of HIV-1 vaccine candidates and to predict immunogenicity in humans. In addition, non-human primates are the only animal model in which efficacy can be assessed by utilizing the HIV-1/SIV chimeras termed SHIVs, and correlates of protections can therefore be defined [18]. Therefore, in the AVIP program, evaluation of novel vaccine candidates based on the combination of Tat and Δ V2 Env is planned.

To this aim adult cynomolgus monkeys (*M. fascicularis*) will be immunized according to protocols established using pre-existing information from human and animal studies. The follow-up will include clinical monitoring (daily) and laboratory testing (bleedings 4 weeks after each inoculation) for standard blood chemistry and evaluation of specific immune responses. T cell responses will be characterized by evaluating (i) the cellular phenotype by FACS analysis, (ii) antigen-driven proliferation, (iii) production of IFN γ , IL-2 and IL-4 by ELISpot and/or intracellular staining, (iv) antigen-specific CTL, (v) CD8-non-cytolytic-antiviral activity, and (vi) CC-chemokines production. Humoral responses will be analyzed by measuring antigen-specific binding Ab titers. The presence of neutralizing Ab will be also assessed against the challenge virus and a panel of HIV primary isolates of different clades to determine the breadth of Ab response.

A special attention will be devoted to assess the efficacy. The challenge will be performed after the last immunization, in a time frame (2 months) suitable to avoid interference by the vaccine-induced immune activation, and with a proper (upon rigorous *in vivo* titration) MID₅₀ infectious dose of a pathogenic SHIV. The control groups will consist of animals receiving the adjuvant alone and naïve animals. The post-challenge follow-up will include multiple bleedings (weeks 0, 2, 4, 8, 12, 16 and 24) for virological (antigenemia, viral load, proviral DNA, virus isolation, cytoviremia, seroconversion) and immunological (see above) studies. Reduced or absent signs of viral replication and lower decline or normal CD4 cell counts in comparison to naïve monkeys will be considered as protection endpoints. Although the checkpoint will be at 2–3 months after the challenge, vaccinated monkeys will be followed for at least a year to evaluate the consequences of vaccination on disease progression.

4.3. Phase I clinical testing

Within our clinical plan, we are developing a novel combined vaccine based on Tat and Δ V2 Env to be tested in phase

I preventive and therapeutic trials in Europe, suitable for future testing in phase II/III trials in developing countries.

Although the development of an effective prophylactic vaccine for HIV remains a paramount goal, many millions of people throughout the world are already infected with HIV and affordable, non-toxic, immunotherapy that induces or re-instates long-lasting, robust, protective immune responses is needed. A direct comparison of vaccines containing distinct combinations of regulatory and structural HIV genes/products in HIV-positive and HIV-negative individuals has never been attempted. Therefore, the vaccine candidate will be administered parenterally to a) eligible HIV-1 negative volunteers at low risk for infection; and to b) eligible HIV-positive patients, in whom viraemia is controlled by drugs (either protease inhibitors-based or non-nucleoside reverse transcriptase inhibitors [NNRTI]-based regimens administered in chronic disease). The primary endpoint of clinical trials will be safety, the secondary endpoint will be immunogenicity.

Safety evaluation will include the assessment of local and systemic reactions, as well as extensive monitoring of hematological and biochemical parameters. In the preventive trials, all individuals will be tested regularly for signs of HIV infection. Specific ELISA for the prescreening of the seroconverted versus vaccinated subjects among the immunized volunteers will be developed, validated and used throughout the studies. Suspected seroconversion will be confirmed by PCR analysis. In the trials involving HIV-positive subjects quantification of plasma viremia and CD4+ T cell numbers will be performed at each time point, since viral rebound and CD4+ T cell decline will provide important endpoints.

5. Conclusions

To date, the promising results from preclinical testing of Tat or Δ V2 Env as single vaccine candidate suggest that the combination of both proteins may lead to a vaccine with superior efficacy. Clinical testing of the single candidates is close to completion, providing a valuable asset to speed up evaluation in phase I trials. Intensive preclinical testing is presently ongoing in order to define critical features of the combined vaccine, such as formulation, dose, route of inoculation, schedule of administration, ensuring the best safety, immunogenicity and efficacy profile. Meanwhile, preparation for clinical trials is actively pursued both in developed and developing countries.

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