Journal of NeuroVirology, 8(suppl. 2): 134–137, 2002
 ISSN 1355–0284/02 $12.00+.00
DOI: 10.1080/13550280290101102

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Conditional virus replication as an approach to a safe live attenuated human immunodeficiency virus vaccine

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Despite intensive efforts, no safe and effective vaccine has been developed for the prophylaxis of human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS). Studies with the simian immunodeficiency virus (SIV)/macaque model demonstrated that live attenuated viruses are the most effective vaccines tested thus far. However, due to ongoing low-level replication of the attenuated virus and the error-prone replication machinery, the attenuated virus may regain replication capacity and become pathogenic. We therefore designed a novel vaccine strategy with an HIV-1 virus that replicates exclusively in the presence of the nontoxic effector doxycycline (dox). This was achieved by replacement of the viral TAR-Tat system for transcriptional activation by the *Escherichia coli*–derived Tet system for inducible gene expression. This designer HIV-rtTA virus replicates in a strictly dox-dependent manner and may represent an improved vaccine strain because its replication can be turned on and off at will. Spontaneous virus evolution resulted in optimization of the components of the Tet system for their new function to support virus replication in human cells. The optimised Tet system may be of particular use in other applications such as inducible expression of gene therapy vectors in the brain. *Journal of NeuroVirology* (2002) **8(suppl. 2),** 134–137.

Keywords: AIDS; doxycycline; HIV; Tet system; vaccine

Live attenuated HIV as a vaccine

The development of a prophylactic human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) vaccine is the best hope for containment of the current pandemic. Most efforts for developing a vaccine focus on immunization with one or several virus proteins that are either directly injected or that are delivered by non-HIV viral vectors or plasmid DNA (reviewed in Sutter and Haas, 2001; Mascola and Nabel, 2001; Robinson, 2002; Smith, 2002). However, there are serious doubts about the efficacy of such vaccines to induce protective immunity. A live attenuated virus appears to be a more potent candidate for eliciting a protective humoral and cellular immune response. The idea of a live attenuated virus vaccine is that the nonpathogenic virus replicates to a limited extent and thereby elicits a potent immune response that protects against subsequent infection with the wild-type pathogenic virus. HIV-1 can be attenuated by deletion or mutation of genes or regulatory elements. The HIV-1 virus encodes five genes that are essential for replication and four accessory genes that are dispensable in particular experimental settings (Figure 1, *top*). These accessory genes have been the major target for attenuation and have been deleted from the viral genome, either individually or in combination (Kestler *et al*, 1991; Gibbs *et al*, 1994; Guan *et al*, 2001). Most research on the development of a live attenuated HIV vaccine

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Vaccine research within the Berkhout laboratory is sponsored by the Technology Foundation STW (applied science division of NWO and the technology program of the Ministry of Economic Affairs, Utrecht, the Netherlands), the Dutch AIDS Fund (AIDS Fonds, Amsterdam, the Netherlands), and the National Institutes of Health (NIH, Bethesda, USA).

Received 16 August 2002; accepted 19 August 2002.

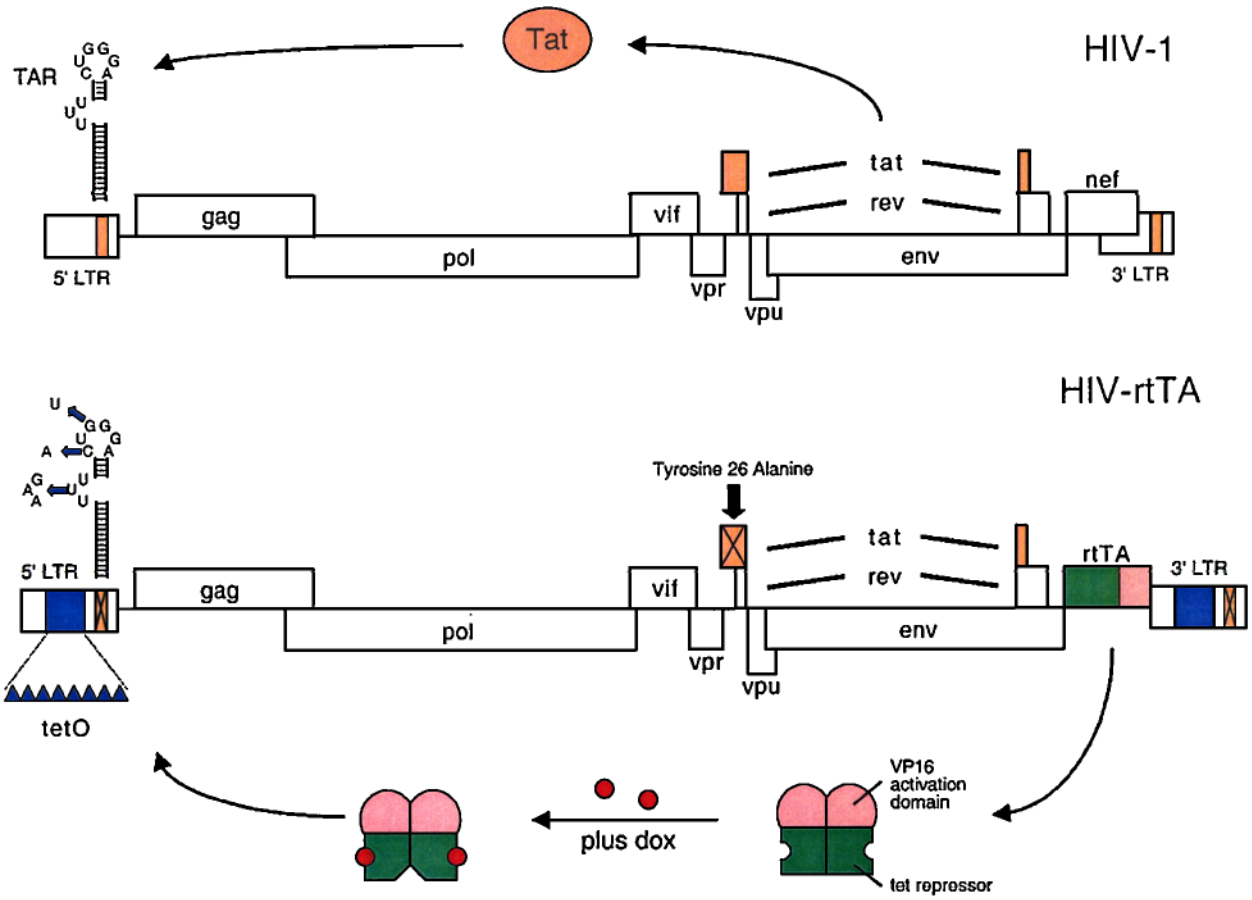

Figure 1 The genomes of wild-type and dox-dependent HIV-1. The HIV-1 genome encodes five genes that are essential for viral replication (gag, pol, env, tat, rev) and four accessory genes (vif, vpr, vpu, nef). The coding region is flanked by long terminal repeats (LTRs) with regulatory signals that are essential for replication. In wild-type HIV-1 (*top*), gene expression and replication are controlled by the viral Tat protein that binds to the TAR hairpin at the 5′ end of the nascent RNA transcript. In the dox-dependent HIV-rtTA virus (*bottom*), the Tat protein and its TAR binding site were inactivated by mutation and functionally replaced by the regulatory elements of the inducible Tet system. The gene encoding the transcriptional activator rtTA, a fusion protein of the *Escherichia coli* TetR protein and the activation domain of the herpes simplex virus VP16 protein, was inserted in place of the 3′-terminal nef gene. Eight copies of the tetO binding site were inserted in the LTR promoter. Binding of dox to rtTA induces a conformational switch that triggers binding to the tetO motifs. Binding of rtTA to tetO activates transcription of the viral genome. Thus, viral gene expression and replication is now controlled by the rtTA protein, and is off in the absence of dox and can be turned on by administration of dox.

has focused on the experimental model system of the pathogenic simian immunodeficiency virus (SIV) of rhesus macaques. Monkeys vaccinated with certain deletion mutants of SIV can efficiently control replication of pathogenic challenge virus strains (reviewed in Desrosiers, 1998; Johnson, 1999; Mills, *et al*, 2000). However, there is accumulating evidence that the attenuated virus can regain replication capacity (Berkhout *et al*, 1999) and cause disease in a minority of the vaccinated individuals (Baba *et al*, 1995, 1999; Deacon *et al*, 1995). Further reduction of the viral replication capacity through progressive deletion or mutation of genes or regulatory elements will minimize the pathogenicity of the vaccine strain, but will also reduce the immunogenicity of the virus and thus the efficacy of vaccination (Lohman *et al*, 1994; Wyand *et al*, 1996; Johnson *et al*, 1999). These results demonstrate the genetic instability and evolutionary capacity of attenuated SIV/HIV strains. Upon vaccination with a live attenuated SIV/HIV virus, the virus is not cleared. Due to ongoing low-level replication of the vaccine strain and the error-prone replication machinery, fitter virus variants will continuously be generated and selected. Because some variants may eventually become pathogenic, live attenuated virus vaccines are considered unsafe for use in humans.

The doxycycline-dependent HIV-rtTA virus

We designed a novel vaccine strategy in which viral replication can be switched off after vaccination to prevent the restoration of virulence. In wild-type and

live attenuated HIV, transcription of the viral genome is controlled by the viral Tat protein that binds to the TAR hairpin at the 5′ end of the nascent RNA transcript (Berkhout *et al*, 1989) (Figure 1, *top*). Translation of the unspliced and spliced RNA transcripts results in the production of all viral proteins, including Tat. Viral gene expression and replication is thus controlled by a constitutive autoregulatory loop. We constructed a virus variant in which gene expression and replication of the virus is not constitutive, but inducible by an antibiotic (Verhoef *et al*, 2001). In this virus, the Tat protein and its TAR-binding site were inactivated by mutation and functionally replaced by components of the tetracycline-inducible gene expression system (Figure 1, *bottom*). This Tet system is based on two elements of the *Escherichia coli* tet operon: the tetracycline-inducible repressor protein (TetR) and the tet operator (tetO) DNA-binding site (Freundlieb *et al*, 1997; Baron and Bujard, 2000). In this system, the transcriptional activator is the rtTA protein, a fusion protein of TetR and the activation domain of the herpes simplex virus VP16 protein. The activity of rtTA is fully dependent on the antibiotic doxycycline (dox). Binding of this nontoxic and selective effector molecule to rtTA induces a conformational switch. The rtTA protein can now bind to the tetO sequence and activate transcription of a downstream-positioned gene. Thus, gene expression is off in the absence of dox and can be turned on by the administration of dox. In the HIV-rtTA virus, the gene encoding rtTA was inserted in place of the 3′-terminal nef gene, and 8 copies of tetO were introduced in the long terminal repeat (LTR) promoter (Figure 1, *bottom*). This HIV-rtTA virus does not replicate in the absence of dox (Verhoef *et al*, 2001). Administration of dox induces transcription of the viral genome, expression of the viral proteins, and replication of the virus. The lack of replication of the HIV-rtTA vaccine candidate in the absence of dox will prevent the evolution towards a pathogenic virus.

Optimization of the HIV-rtTA virus

Our approach to construct a dox-dependent virus was more successful than similar projects of other groups (Xiao *et al*, 2000; Smith *et al*, 2001), but it is obvious that the initial HIV-rtTA virus replicates poorly compared to wild-type HIV-1 (Verhoef *et al*, 2001). We anticipated that viruses with improved replication capacity might evolve during prolonged culturing of the virus (Klaver and Berkhout, 1994). Improved replication might result from repair of the original Tat-TAR system by reversion of the introduced mutations. In this scenario, the components of the rtTA-tetO system will become redundant and might be lost by mutation or deletion. This evolutionary route is not likely because we introduced multiple inactivating mutations in the Tat protein and TAR hairpin. Alternatively, the components of the introduced Tet system, which are largely derived from *E. coli*, might be optimized to support virus replication in human cells. We followed the evolution of HIV-rtTA in multiple cell culture infections, and analyzed the status of the old Tat-TAR axis and the new rtTA-tetO system. In all viruses examined, the introduced mutations in the Tat and TAR were maintained, demonstrating that the original Tat-TAR system is not repaired. Furthermore, all viruses were found to maintain the introduced rtTA gene and the tetO elements. As anticipated, we did see changes in the tetO LTR promoter region and the rtTA protein. A rearrangement in the tetO LTR promoter region was observed in several independent virus cultures (Marzio *et al*, 2001). This rearrangement resulted in a deletion of six of the original eight tetO motifs, followed by a further deletion of 14 or 15 nucleotides in the spacer between the two remaining tetO elements. Interestingly, the final conformation of the tetO elements resembles the conformation of these elements in the *E. coli* Tn10 tet operon. This rearrangement did greatly improve replication of HIV-rtTA. Strikingly, the transcriptional activity of this evolved 2Δ-tetO LTR promoter is lower than that of the original 8-tetO LTR promoter, but mimics the activity of the wild-type HIV LTR promoter (Marzio *et al*, 2002). These results demonstrate that HIV requires a fine-tuned level of transcription for efficient replication. We also observed amino acid changes in the rtTA protein that improve the replication capacity of the virus. For instance, we selected an rtTA variant with a mutation in the dox-binding site that greatly improves dox sensitivity and maximum activity of the protein. This HIV-rtTA variant requires much less dox for optimal virus replication (manuscript in preparation). These results underline the genetic flexibility of HIV-1, which can be exploited for the functional adaptation of the dox-inducible expression system and to further improve the HIV-rtTA virus. We are currently trying to adapt HIV-rtTA to dox-like compounds that lack antibiotic activity. This will be another major step forward in the design of an effective and safe vaccine based on a drug-dependent HIV-1 variant. The Tet system for inducible gene expression is widely used in many experimental set-ups, for instance in transgenic animals and gene therapy approaches. The optimized rtTA-tetO reagents that are obtained through virus evolution will be useful in several of these applications. For example, improved dox sensitivity will allow the use of the Tet system in tissues such as the brain, where relatively low dox concentrations are reached.

Vaccination with HIV-rtTA

The conditional-live HIV-rtTA virus replicates only in the presence of dox. Vaccination with this virus in the presence of dox will activate an immune response. The period of active virus replication that is needed to mount protective immunity is not known,

but may be as short as 1 day (Lifson *et al*, 2001). Subsequent withdrawal of dox switches the virus off and blocks viral evolution and the appearance of pathogenic viruses. If needed, virus replication can be turned on at a later moment as booster vaccination by additional administration of dox (Berkhout *et al*, 2002). Repeated antigenic stimulation may be critical to preserve immunological memory. We realize that this approach has to be vigorously tested for safety and efficacy. For this reason, a similar SIV-rtTA vaccine virus will be constructed for testing in rhesus macaques.

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