Development and characterization of a new single cycle vaccine vector in the simian immunodeficiency virus model system

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Received 18 July 2007; returned to author for revision 14 August 2007; accepted 20 September 2007
Available online 26 November 2007

Abstract

We have developed a new single cycle lentiviral vector, SIVsmH4i-SC27.1, as a potential SIV/HIV-1 vaccine candidate. This viral vector is capable of expressing all of the SIV gene products but is limited to one round of infection. The vector was created by mutating 27 codons dispersed among the viral vif, env, and nef genes to block protein function, attenuate viral replication/infectivity, and reduce the ability of the virus to manipulate the host immune system. To complement the env and nef replication defects, SC27.1 was pseudotyped with the VSV G glycoprotein to allow particle entry. The vif mutation was complemented by producing particles from an APOBEC3G-negative cell line, and the Vif protein defect was validated by showing that the single cycle virus lost most of its infectivity when particles were produced in presence of APOBEC3G. To deal with the problem of an antibody response to the VSV G protein in a vaccination strategy, two additional serotypes of the VSV G protein were used to create pseudotyped virus particles, and we observed no cross-neutralization activity for two of the pseudotyped particles with a potent neutralizing antiserum to one of the VSV G proteins. We detected moderate inhibition of infectivity in normal human and macaque sera, especially to the New Jersey serotype of VSV G, but as a heat sensitive activity, presumably complement mediated. These particles can be used in a prime-boost strategy to determine if a single cycle lentiviral vaccine vector capable of expressing all of the viral gene products holds promise in inducing immunity and protection to an SIVsm challenge.

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Keywords: Lentiviral vector; SIV; HIV-1 vaccine; VSV G

Introduction

Second generation HIV-1 vaccine candidates are in or near human testing. These include DNA vaccines, protein boosts, and single cycle virus vectors for a variety of poxviruses, alphaviruses, adenovirus, adeno-associated virus, and more (see http://www.iavireport.org/trialsdb/). A logical extension of this concept is a single cycle retrovirus that can serve as its own vector for the delivery of the viral gene products. This approach is distinguished from an attenuated virus that goes through multiple rounds of replication but with the attendant potential for evolution to increased pathogenesis. Several designs of single cycle vectors are in development with some initial promising results (Baliga et al., 2006; Evans et al., 2004, 2005; Kuate et al., 2003).

There are potential concerns regarding integrating vectors that were realized with the induction of cancer through insertional activation in a gene therapy trial that employed a murine leukemia virus (MLV) vector (Hacein-Bey-Abina et al., 2003). While this particular example is difficult to generalize due to the possible contribution of the gene insert in the retroviral vector (discussed in McCormack and Rabbits, 2004), these cases do serve to reinforce the idea that the potential effects of the vector need to be considered.

Lentiviruses are intrinsically different in their biology compared to oncogenic retroviruses, including MLV, where the potential for insertional activation of cellular oncogenes is well known. There is no evidence that any lentivirus causes tumors in spite of chronic
infections with persistent replication (and integration). While the reason for the difference in the disease types among different lineages of retroviruses is not known, one possible difference that could provide a mechanistic explanation is the use in lentiviruses of trans acting transcriptional factors, like Tat for HIV-1. The Tat protein targets the TAR sequence on short viral transcripts, localizing P-TEFs (which contains CDK9/Cyclin T1) to the viral transcription unit to promote processive RNA synthesis through phosphorylation of the C-terminal domain of RNA polymerase II (reviewed in Rice and Herrmann, 2003). This mechanism is distinct from the transcriptional enhancement of oncogenic retroviruses, which attract transcription factors to the LTR that can remodel chromatin regionally (Nusse et al., 1984; Short et al., 1987). It is also distinct from HTLV-1, which uses its transcriptional transactivator protein Tax as an adaptor protein to promote assembly of a transcription factor complex but without the localization effect of the Tat-TAR interaction (Kwok et al., 1996; Giebler et al., 1997; Lu et al., 2002). Thus, the HIV-1 transactivation protein appears to limit its transcriptional impact on the host genome to the provirus, with greatly reduced potential to impact cellular genes including oncogenes.

In this study, we developed a defective genome derived from the SIVsmH4i infectious and pathogenic molecular clone (Hirsch et al., 1989a,b). The single cycle vector carries 71 the SIVsmH4i infectious and pathogenic molecular clone impact cellular genes including oncogenes. The viral Nef protein is a multifunctional protein whose functions include down modulation of the immune response through MHC class I internalization, down modulation of the viral receptor CD4, increases in viral infectivity, and interaction with PakII (Wei et al., 2003). Five conserved sequence motifs in the nef gene were targeted for mutagenesis. First, three of the positions encoding amino acids that function in myristylation (MGXXXSK to MAXXXXAA) were mutated to inhibit the myristylation reaction, Nef membrane targeting, CD4 down-regulation, and virion infectivity (Harris and Coates, 1993; Geyer et al., 1999, 2001). Second, three conserved codons encoding acidic amino acids were mutated to encode Arg, a mutation that affects retention of MHC class I in the golgi (Mangasarian et al., 1999). Third, two Pro codons in the SH3-binding PXXP motif were deleted to disrupt the interaction between Nef and signaling pathways (Xu et al., 1999; Fackler et al., 1999; Saksela et al., 1995). Fourth, two Arg codons were mutated, with these residues having been reported to be involved in Pak activation (Renkema et al., 1999; Fackler et al., 2000). Finally, two other pairs of conserved residues (HXB number 123, 124 DW to AA, and 144, 147 KXXP to AXXV) were mutated based on the assumption that sequence conservation implies participation in an important function. In total, 14 codons encoding conserved residues in Nef were mutated (Table 1). The mutations were incorporated into subclones of the viral genome which were then reassembled using convenient restriction enzyme sites (Fig. 1). In total, 27 codons dispersed in env, nef, and vif were chosen for mutagenesis with three or more mutations in each gene that were designed to allow protein expression as an immunogen but block protein function (Table 1).

We took advantage of the large amount of information known about the function of the viral Env protein to make numerous targeted mutations to ablate multiple functions. We first mutated three Arg residues to Ala upstream of the gp120/gp41 cleavage site to disrupt gp120/gp41 processing (Bosch and Pawlita, 1990). We next mutated three Phe residues to Ser in the fusion peptide domain just downstream of the gp120/gp41 cleavage site to inhibit its activity in membrane fusion. In addition, two Trp residues were mutated to Ala in the C-terminal heptad repeat of gp41, to disrupt the coiled-coil intermediate of Env-mediated membrane fusion (Salzwedel et al., 1999). Finally, two stop codons were introduced at the upstream boundary of the gp41 cytoplasmic tail domain to create a truncated form of the Env protein, to improve Env surface expression (Spies and Compans, 1994; Edwards et al., 2002). In total, 10 codons were mutated in env directed at four distinct determinants of Env protein function (Table 1).

The viral protein Vif mediates the degradation of the cellular proteins APOBEC3G/F to prevent their encapsidation in the virus particle, which would otherwise lead to deamination of newly synthesized viral DNA in a subsequent round of infection (Sheehy et al., 2002; Harris et al., 2003; Mangeat et al., 2003). We mutated the highly conserved motif YFPC to AAPA (Table 1). The YF/AA mutations and the C/A mutation have each been reported to the lower infectivity of HIV-1 to between 1% and 15% (Simon et al., 1999).

Results

Design and construction of the single cycle vector SC27.1

We mutated 27 codons, dispersed among three viral genes, in the infectious SIVsm molecular clone of SIVsmH4i (Hirsch et al., 1989b). We first generated subgenomic clones of SIVsmH4i to allow simplified protocols for introducing the mutations then regenerated the full-length clone. The mutations were selected such that the final viral genome would be able to express all of the viral gene products, and would be able to create a virus particle that, when pseudotyped, could initiate only one round of replication. The viral genes env, vif, and nef were chosen for mutagenesis with three or more mutations in each gene that were designed to allow protein expression as an immunogen but block protein function (Table 1).

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genome, the probability of reversion via the hypermutation pathway to a functional state of replication appears remote.

Production and titration of single-cycle immunodeficiency viruses

The goal of the design of SC27.1 was to ablate particle infectivity at the entry step with the mutations in the Env protein, reduce particle infectivity and the ability to modulate the immune response with the mutations in the Nef protein, and make the replication cycle susceptible to the mutagenic effects of APOBEC3G/F with the mutations in the Vif protein. However, to make infectious particles it was necessary to complement several of these defects, at least during particle production. This was done in two ways. First, the Vif defect was overcome by producing virus in cells that do not express APOBEC3G, and we chose 293T cells (Sheehy et al., 2002) for producing virus particles after transfection of the full-length (mutated) viral DNA. Second, we complemented both the Env defect and the Nef virion infectivity defect by pseudotyping the virus particle with the VSV G protein by including a VSV G expression vector in the transfection (Reiser et al., 1996; Naldini et al., 1996; Aiken, 1997; Luo et al., 1998; Chazal et al., 2001). In this way, we were able to produce infectious virus particles using the unmutated Gag, Pro, and Pol proteins to make particles that packaged a viral genome that did not encode functional Env, Vif, or Nef proteins.

<table>
<thead>
<tr>
<th>Table1 Mutations in SC27.1</th>
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<tbody>
<tr>
<td><strong>Gene</strong></td>
<td><strong>Motif s to be changed</strong></td>
</tr>
<tr>
<td>env</td>
<td>(503)RRxxxxxxxRxKR</td>
</tr>
<tr>
<td></td>
<td>(514)FxxxFxxxx</td>
</tr>
<tr>
<td></td>
<td>(628)WxxW</td>
</tr>
<tr>
<td>vif</td>
<td>(710)QG</td>
</tr>
<tr>
<td>nef</td>
<td>(102)YFxC</td>
</tr>
<tr>
<td></td>
<td>(140)DW</td>
</tr>
<tr>
<td></td>
<td>(160)KxxP</td>
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<td></td>
<td>(132)RR</td>
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<td></td>
<td>(140)DW</td>
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<tr>
<td></td>
<td>(160)KxxP</td>
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</tbody>
</table>

Fig. 1. Box cartoon of the genome showing restriction sites and engineered mutations. Unique restriction sites used to create the construct are indicated. The approximate positions of the mutated functional domains dispersed among the *vif*, *env* and *nef* genes are labeled as *.

Fig. 1. Box cartoon of the genome showing restriction sites and engineered mutations. Unique restriction sites used to create the construct are indicated. The approximate positions of the mutated functional domains dispersed among the *vif*, *env* and *nef* genes are labeled as *.
The SIVsmH4i-SC27.1 clone and a VSV G(I) expression vector were cotransfected into 293T cells and the virus particles were collected in the supernatant medium. An aliquot of the medium was subjected to centrifugation to concentrate the virus particles. We used two independent methods to determine the mass of virus produced by the transfection. First, we used an ELISA to measure the amount of viral p27 capsid (CA) protein present in the medium after transfection and then after further purification. The ELISA p27 titer was between 3 and 11 μg/ml in the supernatant medium and between 31 and 310 μg/ml after a 36-fold concentration (Table 2). Similar levels of p27 were produced after transfecting either the parental SIV DNA clone or the SC27.1 plasmid without the pseudotype expression vector. These results were further confirmed by examining viral protein mass by silver staining after polyacrylamide gel electrophoresis under denaturing and reducing conditions. As a control, we used a highly purified and quantified virus preparation (SIVmac239/SUPT1-CCR5 CL.30) to generate a visual standard curve. The p27 titer was estimated to be between 20 and 100 μg/ml for the concentrated particles derived by transfection of the mutant (Fig. 2) and parental plasmid clones (data not shown). Similar results were obtained when infectivity was monitored from production through purification (Table 3). These results document the production and infectivity of the SC27.1 construct as infectious particles.

Characterization of viral protein expression from SIVsmH4i-SC27.1 after transfection and infection

The presence of viral proteins encoded in the SC27.1 vector produced by transfection of 293T cells was characterized by Western blot analysis and compared to the fully infectious parental clone. Both the viral Gag and Env proteins could be detected in the lysate of the transfected cells (Fig. 3). Due to the cytoplasm tail truncation and cleavage site mutations, the Env in the SC27.1 lysate was smaller than the full-length gp160 Env precursor but larger than the processed gp120 SU protein (Fig. 3A, compare lanes 2 and 3). The Gag proteins were expressed at levels comparable to the level of expression from the parental clone (Fig. 3B), although when analyzed with a polyclonal antibody we have consistently observed modestly reduced processing of Gag when expressed from the SC27.1 vector (Fig. 3C). Supernatant virus was concentrated by pelleting through a 20% sucrose cushion then analyzed for particle-associated Gag and Env proteins (Fig. 3D). Gag protein from cells transfected with the SC27.1 construct could be detected with or without the inclusion of the VSV G expression vector.

Table 2

<table>
<thead>
<tr>
<th>Transfected plasmid</th>
<th>Supernatant</th>
<th>Purified particles</th>
<th>Recovery rate (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>p27 con. (μg/ml)</td>
<td>Volume (ml)</td>
<td>Total p27 (μg)</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
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</tr>
<tr>
<td>SC27.1/pCIVSV G(Ch)*</td>
<td>3.6</td>
<td>198</td>
<td>712.8</td>
</tr>
<tr>
<td>SC27.1/pCIVSG G(I)*</td>
<td>3.5</td>
<td>198</td>
<td>693.0</td>
</tr>
<tr>
<td>SC27.1/pCIVSG G(NJ)*</td>
<td>4.5</td>
<td>198</td>
<td>883.1</td>
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<tr>
<td>SIVsmH4i*</td>
<td>3.8</td>
<td>15</td>
<td>57.0</td>
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<tr>
<td>SC27.1</td>
<td>4.0</td>
<td>198</td>
<td>672.0</td>
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<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>SC27.1/pCIVSV G(Ch)*</td>
<td>4.1</td>
<td>198</td>
<td>823.7</td>
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<tr>
<td>SC27.1/pCIVSG G(I)*</td>
<td>11.4</td>
<td>198</td>
<td>2257.2</td>
</tr>
<tr>
<td>SC27.1/pCIVSG G(NJ)*</td>
<td>4.2</td>
<td>198</td>
<td>833.6</td>
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</table>

* Transfected plasmids molar ratio molar ratio SC27.1: pCIVSV G = 1:2, 10.5 μg SC27.1 and 9.5 μg pCIVSV G, total 20 μg DNA.

b Transfected plasmids molar ratio SC27.1: pCIVSV G = 1:5, 10.5 μg SC27.1 and 23.5 μg pCIVSV G total 34 μg DNA.
vector (Fig. 3D, compare lane 2 to lanes 4, 5, and 6), and the released particles still retained some under-processed Gag protein relative to the parental virus (Fig. 3D, compare lanes 2 and 3). The Env protein was detected in the parental SIV particles but not in the SC27.1 particles either with or without the VSV G pseudotype expression vector (Fig. 3D), suggesting the mutated form of the Env protein failed to package into the particles. We do not know the cause of the under-processing of Gag; it is possible this is an effect of an absence of the cytoplasmic domain of Env interacting with MA, although we have not tested this possibility directly.

The above experiment examined the expression of viral proteins after transfection of the mutagenized genomic clone; we also wanted to document the expression of the viral proteins after infection. TZM-BL cells were infected with VSV G-pseudotyped SC27.1 particles or with the parental SIV. Proteins were radioactively labeled at steady state (to distinguish input virus from newly synthesized viral proteins), and the cell lysate and virus-containing supernatant medium were collected at 48 h post-infection. Viral proteins were analyzed using immunoprecipitation and denaturing gel electrophoresis. Cell-associated expression of Gag p27 was apparent in both pseudotype virus- and parental virus-infected cells (Fig. 4A). The non-cleaved and truncated form of Env was detected in the pseudotype SC27.1-infected cells lysates (Fig. 4B). Particle-associated Gag p27 protein was detected in particles pelleted from the supernatant of the cells infected with pseudotyped SC27.1 or with the parental virus (Fig. 4C). These results show that the SC27.1 vector is able to go through a round of infection then direct subsequent gene expression.

Validation of the phenotypes of the mutated proteins

To validate whether the vif mutations disrupted Vif protein function, 293T cells (which do not express APOBEC3G) were transfected with the SC27.1/VSV G(I) plasmids alone or with an APOBEC3G expression vector at 4 different molar ratios. As a control, the parental infectious clone SIVsmH4i was similarly

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Table 3

<table>
<thead>
<tr>
<th>Transfected plasmid</th>
<th>Supernatant titer (BFU/ml)</th>
<th>Total volume (ml)</th>
<th>Total BFU</th>
<th>Purified particles titer (BFU/ml)</th>
<th>Total volume (ml)</th>
<th>Total BFU</th>
<th>Recovery rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC27.1/pCIVSV G(Ch)*</td>
<td>6.34E+4</td>
<td>198</td>
<td>1.26E+7</td>
<td>6.14E+5</td>
<td>3.5</td>
<td>2.15E+6</td>
<td>16.9%</td>
</tr>
<tr>
<td>SC27.1/pCIVSG G(I)*</td>
<td>6.64E+4</td>
<td>198</td>
<td>1.31E+7</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>SC27.1/pCIVSG G(NJ)*</td>
<td>7.2E+5</td>
<td>15</td>
<td>2.15E+6</td>
<td>1.01E+7</td>
<td>3.5</td>
<td>3.53E+7</td>
<td>72.6%</td>
</tr>
<tr>
<td>SC27.1*</td>
<td>0</td>
<td>198</td>
<td>0</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC27.1/pCIVSV G(Ch)b</td>
<td>2.64E+5</td>
<td>198</td>
<td>5.22E+7</td>
<td>7.55E+6</td>
<td>3.5</td>
<td>2.64E+7</td>
<td>50.6%</td>
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<tr>
<td>SC27.1/pCIVSG G(I)b</td>
<td>4.15E+5</td>
<td>198</td>
<td>8.21E+7</td>
<td>1.87E+7</td>
<td>3.5</td>
<td>6.55E+7</td>
<td>79.7%</td>
</tr>
<tr>
<td>SC27.1/pCIVSV G(NJ)b</td>
<td>2.19E+5</td>
<td>198</td>
<td>4.34E+7</td>
<td>4.04E+5</td>
<td>3.5</td>
<td>1.41E+6</td>
<td>86.5%</td>
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* Transfected plasmids molar ratio SC27.1: pCIVSV G=1:2, 10.5 μg SC27.1, and 9.5 μg pCIVSV G, total 20 μg DNA.

b Transfected plasmids molar ratio SC27.1: pCIVSV G=1:5, 10.5 μg SC27.1, and 23.5 μg pCIVSV G total 34 μg DNA.

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Fig. 3. Western blot analysis of viral protein expression after transfection of producer cells. 293T cells were transfected with plasmids as indicated below. Panels A and B are the analysis of cell lysates from the transfected cells. Viral particles were prepared from the supernatant of transfected cells and purified by centrifugation through a sucrose cushion. Panels C and D are the analysis of viral particles produced from transfected cells. Lane 1, mock; lane 2, SC27.1; lane 3, SIVsmH4i; lane 4, SC27.1/ pCIVSV G(Ch); lane 5, SC27.1/pCIVSV G(I); lane 6, SC27.1/pCIVSV G(NJ). The western blots were probed with SIV anti-Env monoclonal antibody 36D5 (Panel A), SIVmac251 anti-Gag monoclonal antibody (Panels B and C) or serum pooled from several SIVsm-infected macaques (Panel D).
tested. The viruses generated were harvested at 48 h then used to infect TZM-BL cells to measure their infectivity (Fig. 5). The infectivity of the SC27.1 virus was reduced to 25% in the presence of the APOBEC3G expression vector at a plasmid molar ratio of 10:1 (APOBEC3G: SC27.1), yet the unmutated parental virus showed essentially no change in infectivity. Increasing the APOBEC3G plasmid to a molar ratio to 20:1 dropped the SC27.1 virus infectivity to less than 10% while the parental virus maintained at least 75% infectivity. These results confirm a previous study showing that these equivalent vif mutations significantly lowered the infectivity of HIV-1 (Simon et al., 1999).

As shown above, the mutations in the env gene resulted in an unprocessed and truncated Env protein that was defective for virion incorporation and for particle infectivity. We examined whether the mutant Env protein could be expressed on the surface of the cell. As shown in Fig. 6A, there is enhanced surface expression of the mutated SC27.1 Env protein compared to the unmutated gp160 protein. An unmutated Env protein with the C-terminal truncation showed similar enhanced surface expression, suggesting the other mutations in the SC27.1 Env protein did not significantly affect its transport to the cell surface. The enhanced surface expression of the truncated protein was expected as the cytoplasmic domain contains endocytosis signals that reduce surface expression of the full-length Env protein (Rowell et al., 1995; Sauter et al., 1996; Boge et al., 1998; Bowers et al., 2000; Wyss et al., 2001). Given that the mutated Env protein was expressed on the cell surface but not incorporated into virus particles, we sought other evidence for the effect of the mutations. As shown in Fig. 6B, the infection of TZM-BL cells with the parental virus leads to the formation of syncytia as the infected cell expresses the Env protein and mediates fusion with nearby cells expressing the receptor and coreceptor. However, cells infected with SC27.1 did not induce syncytia with the adjacent cells. This documents the loss of fusion capacity anticipated with the mutations that block Env processing, disrupt the hydrophobic nature of the fusion peptide, and change key residues involved in the six helix bundle formation.

We attempted to document the inability of the mutant Nef protein to down-regulate surface expression of MHC class I, however neither the mutant nor the parental Nef proteins could be shown to down regulate the class I protein (data not shown) thus precluding a measure of the effect of the mutations. We did not document protein expression of Vif or Nef due to a lack of antibody reagents.

Neutralization properties of the pseudotyped SC27.1 particles

An immune response to the vaccine vector is likely to limit the utility of a boost strategy with a homologous vector. To avoid the potential impact of the VSV G surface protein inducing neutralizing antibodies, we made SC27.1 particles pseudotyped with three different VSV G serotypes: G(I), Indiana; G(NJ), New Jersey; and G(Ch), Chandipura. We tested the potential for cross-neutralization between these G proteins by incubating the three pseudotyped virus preparations and the SIV parental virus in...
presence of a dilution series of a VSV G(I) neutralizing rabbit antiserum. As shown in Fig. 7A, the VSV G(I) pseudotyped SC27.1 was neutralized at all dilutions tested (up to 1:6400). In contrast, there was no neutralization of any of the other viruses even at the lowest dilution tested (1:100). These results confirm the serotype classification of the VSV G proteins, even as pseudotypes, and confirm the potential utility of using different VSV G-pseudotyped viruses to avoid the problem of an immune response to the vector in a prime-boost vaccination strategy (Rose et al., 2000).

VSV G pseudotyped lentiviral vectors have been reported to be partially inactivated by human complement (DePolo et al., 1999). To determine the extent to which these VSV G-pseudotyped single cycle vectors were sensitive to human and macaque sera, the three different VSV G protein-pseudotyped viruses and the SIV parent were incubated with 50% normal or heat-inactivated serum. The residual titer was quantified and the results recorded as the percent recovery of infectivity compared to control samples that were not exposed to human or macaque serum. An overall sensitivity to inactivation by both human (Fig. 7B) and macaque (Fig. 7C) serum was observed. The effect was greater with the macaque serum samples, and VSV G(NJ) was more sensitive than the other two serotypes. Inhibition was largely through a heat-sensitive component, presumably complement-mediated, and after heat inactivation of the serum two of the VSV G pseudotypes (CH and I) had their infectivity enhanced by exposure of the serum.

The infectivity of SC27.1 is limited to a single cycle of infection

To analyze whether replication competent virus would be produced by SC27.1-infected cells, we used pseudotyped virus to infect CEM × 174 cells at an MOI of 1 (passage 1), and the cell supernatant was blind passaged onto fresh CEM × 174 cells...
twice (passages 2 and 3). In each passage, the cells were maintained for 2 weeks to assess the appearance of replication virus using a p27 CA ELISA assay. During passage 1, p27 CA was detected at a low level from days 2 to 4 post-infection, due to the input virus and/or the first round of infection. After the first several days, no p27 CA was detected during the remainder of passage 1 or at any point during passage 2 or passage 3. In contrast, the parental SIV produced a rapid increase in p27 and extensive cells death, even when the infecting inoculum was at an MOI of 0.01 (data not shown). These results are consistent with the design of the single cycle virus vector in being limited to a single round of infection and, at least under the conditions tested, being unable to revert to replication competence.

**Discussion**

Several single cycle vectors have been described previously. In one case, changes in the gag-pro-pol frameshift site were used to make the single cycle vector that in the first iteration used the viral Env glycoprotein for entry (Evans et al., 2004, 2005). This virus was able to infect macaques and showed transient levels for circulating virus. Vaccination-challenge studies indicated immunization of macaques with this vector stimulates diverse virus-specific immune responses, and three of four immunized animals had reduced viral RNA loads of 1–3 log in the acute phase of infection after challenge. The use of a VSV G pseudotype with a single cycle vector has been described previously (Tung et al., 1998). In this approach, the pol and env genes were truncated then complemented in trans with a functional pol gene and VSV G. The authors reported no recombinant virus (although this approach would appear to raise the problem of recombination during the generation of virus with the two pol vectors), and they saw reduction in viral RNA load to below the level of detection in three vaccinated animals after challenge. No measures of the immune response were reported. Baliga et al. (2006) inserted a truncated form of cyclin T1 in place of the env gene and, using a VSV G pseudotype, were able to generate immune responses in mice. Finally, in another report of a single cycle vector with an altered tRNA binding site and four accessory gene deletions, and using a VSV G pseudotype strategy, a decrease in peak viremia (but not set point) after challenge with SIVmac239 was seen (Kuate et al., 2003). These preliminary reports are encouraging and support the investigation of this class of vaccine vectors. In designing our single cycle vector, we avoided the full or partial gene deletions that were used in these previous versions as they remove potential immunogenic regions. Also, we used only the multiple mutations in coding regions to limit the vector to a single cycle rather than the more complex designs of the previous versions which allows us to limit the components in producing the vaccine to the vector backbone and the surface protein expression vector for pseudotyping.

At this point a great deal of information is known about the structure-function relationship of all of the viral proteins. Thus, it was possible to create a vaccine vector that was mutated in a subset of the viral genes (vif, env and nef) with mutations designed to disrupt multiple functions of the protein product while still permitting a single round of infection with the subsequent expression of all of the viral proteins as potential immunogens. We were able to accomplish this with a single plasmid representing the SIV genome and a heterologous viral glycoprotein expression vector but without the need of a packaging cell line. The heterologous glycoprotein and the choice of cell lines for producing virus particles allowed us to complement the mutated functions missing in the vaccine vector.

There are two important theoretical concerns that must be addressed when considering a retrovirus single cycle vector. First, retroviruses integrate into the host genome and, although not tested directly, the design of this vaccine vector should permit an integration step during the one round of replication. The phenomenon of insertional activation as a mechanism of tumor induction is well known for certain genera of replication competent retroviruses, including the gammaretroviruses. However, most of the experience with gammaretrovirus vectors in gene therapy trials (analogous to a single cycle vector) showed this risk was minimal. Recently, one trial to introduce the IL2R gamma(c) gene into children with an immunodeficiency using an MLV vector resulted in repeated activation of the LMO-2 gene (Hacine-Bey-Abina et al., 2003). It seems likely the gene insert contributed a second signal in this case, a result not previously observed in other gene therapy trials.

In contrast, no lentivirus, including HIV-1, has been associated with tumor induction in its target cell. HIV-1-induced immunodeficiency is associated with the appearance of tumors caused by the herpesviruses KSHV and EBV, but there is no evidence for increased frequencies of CD4+ T cell leukemias or lymphomas as would be expected if chronic replication put the target cell at risk for insertional activation of cellular oncogenes (Frisch et al., 2001; Dal Maso and Franceschi, 2003), in spite of efforts to implicate HIV infection through such a mechanism (Mack et al., 2003). It is possible that lentiviruses have a replication strategy that includes a weak enhancer regulated by transactivating factors like Tat, and that these factors show a high degree of specificity for activating transcription from the provirus, a mechanism that would not favor regional transcriptional activation (Grassmann et al., 2005). In addition, HIV-1 tends to integrate within genes rather than in flanking regions as do the gammaretroviruses (Schroder et al., 2002; Wu et al., 2003), which would be more compatible with enhancement of expression of an undisrupted gene. However, the specificity of this difference is probably not sufficiently great to account for the striking biological difference of an absence of tumor formation associated with lentiviruses. Also, while the mechanism for transactivation expression of the viral genome is well characterized and requires the cis-acting viral TAR sequence, transcription of cellular genes has been reported to be altered by Tat expression (Marzio et al., 1998; Pumfery et al., 2003), although the mechanism of this effect is not clear. Finally, infection of T cells by HIV-1 is cytotoxic and this may preclude the risk of insertional activation; in reality, the potential of retroviruses to generate deletions during reverse transcription makes it likely that there are may proviral insertions that encode defective virus incapable of inducing cytotoxicity.
The second theoretical concern has to do with the potential for reversion to replication competence and pathogenesis. Reversion of attenuated viruses to a higher level of replication capacity has been observed for both HIV-1 and SIV (Koenig et al., 1995; Ferbas et al., 1996; Goulder et al., 1997; Barouch et al., 2002). However, these examples typically involve the reversion of one mutation. Variants with deletion mutations can still undergo some sequence evolution but replication persists only at low viral RNA loads, although this can lead to pathogenesis (Deacon et al., 1995; Sawai et al., 2000; Alexander et al., 2003). The potential of APOBEC3G/F to induce hypermutation further contributes to the genetic instability of the HIV-1/SIV system (Harris and Liddament, 2004). In designing the SC27.1 vector, we sought to overcome the problem of reversion by targeting multiple functions in multiple genes for mutagenesis. Thus, one or even several fortuitous mutations should not permit reversion to replication competence, which would then provide a system where selective pressure would come into play. Similarly, only a minority of the mutations that we incorporated would be reverted by G-to-A hypermutation, and there is no mechanism that would focus this deleterious event to the mutated codons. In the same way, random mutations would not target the mutated codons and the level of mutagenesis required to revert the designed mutations would be expected to introduce many other detrimental mutations at other sites. Our modest attempt to select for a revertant gave a negative result, although an assessment in an animal model will be a more rigorous test.

The VSV G pseudotype coat will target the vaccine vector into cell types that do not normally get infected by HIV/SIV. Studies of pseudotyped gene therapy vectors in mice have shown that the VSV G glycoprotein directs infection of non-dividing cells when given IV, with infection occurring largely in liver, spleen, and bone marrow cells (Naldini et al., 1996; Pan et al., 2002). A basic limitation of viral vector systems is that the animal can develop neutralizing antibodies to the vector after the first vaccination and these antibodies can prevent subsequent boosting. In the case of VSV G glycoprotein, anti-G antibodies are highly effective at preventing reinfection (Rose et al., 2000). Kuate et al. (2003) found the anti-VSV G antibodies raised in the first inoculation with their single cycle vector blocked a detectable boost following the second infection. To avoid such a limitation, we have generated three VSV G protein pseudotyped single cycle viruses that should allow effective boosting even in the presence of neutralizing antibodies directed against the previous inoculated vectors. The VSV G proteins described here, G(I), G(Ch), and G(NJ) do not elicit cross-neutralizing antibodies to their divergent G proteins (Rose et al., 2000). Furthermore, additional pseudotyping viral attachment proteins could be included in this strategy to permit continual boosting. Finally, the form of the SIV Env protein used in this strategy allows surface expression on the infected cell but is poorly incorporated into the virus particle, potentially allowing induction of a neutralizing antibody response to SIV Env that would not target the boost particles.

The initial HIV-1 vaccine trials have shown no efficacy but more promising vaccine strategies are well into human trials (reviewed in Duerr et al., 2006). At present there is no correlate of protection leaving vaccine development an empirical effort. Thus, there is good reason to continue to expand potential vaccine strategies that may offer distinct advantages.

Materials and methods

Construction of the single cycle vector SIVsmH4i-SC27.1

A 1655-bp fragment (Fragment A, nucleotides 155–1811) that included the left LTR and part of gag gene from the infectious molecular clone SIVsmH4i (GenBank No: X14307) was subcloned into the Zero Blunt PCR-vector (Invitrogen, Cat. K2700-20) after amplification by PCR. The amplicon truncated 155 bp at the left end of the upstream LTR to remove overlap with the mutated regions of the nef gene present in the downstream LTR. An Eagl site was included in the left end, and a polylinker BsrG1-BeII-Clal-BglII-Mul was included in the right end of the fragment to facilitate the subsequent cloning steps. Four other ampiclons representing the downstream portions of the genome were generated guided by convenient restriction enzyme sites to aid in the reassembly of the full-length genome (fragment B, nucleotides 1739–5125, 3385 bp; fragment C, nucleotides 5101–8044, 2914 bp; fragment D, nucleotides 8034–8898, 808 bp; and fragment E, 8842–10241, 1395 bp). Each of the individual ampiclons was subcloned as a blunt-end PCR product.

The subcloned fragments were used as templates for mutagenesis using the Quick-Change mutagenesis protocol where the entire plasmid is resynthesized by PCR using complementary mutagenic primers. After adding mutations to each of the fragments, the genome was reassembled by adding the fragments back sequentially, using the polylinker and unique restriction sites within the genome to build the single cycle vector SC27.1. The reassembled genome was fully sequenced to validate its structure.

Vesicular stomatitis virus glycoprotein (VSV G)

Three serotypes of vesicular stomatitis virus glycoprotein (VSV G) were included in this study. A plasmid containing the Indiana strain glycoprotein [G(I)] in the expression vector pCI was kindly provided by Dr. John Olsen. Clones of two other serotypes of the VSV G gene, from the New Jersey [G(NJ)] and the Chandipura [G(Ch)] strains, were a gift from Dr. John Rose and Dr. Amiya Banerjee, respectively. To put the VSV G(NJ) and the VSV G(Ch) genes into the same expression vector as pCI-G(I) clone, we created an AvrII site after the stop codon of the nef gene from the pCI-G(I) vector, which had been digested with XhoI and AvrII to remove the VSV G(I) insert. The new plasmids were designated pCI-G(NJ) and pCI-G(Ch).
Production of virus

The 293T cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. Cells were seeded at 5×10^6 cells per 150-mm plate the day before transfection. VSV G-pseudotyped virus was prepared by mixing the cloned SC27.1 plasmid and one of the VSV G expression vectors with the FuGENE6 transfection reagent (Roche, Cat. 11814443001) following the manufacturer’s instructions. The transfection mix was removed after 6 h, the cells washed, and 15 ml of serum-free medium added (Invitrogen, Cat. 12338-018). The culture supernatant containing virus particles was harvested 48 h later and either used for infection directly or concentrated by pelleting at 14,000 rpm for 3 h by ultracentrifugation with or without a 20% sucrose cushion (Beckman SW28i); virion pellets were resuspended in PBS. The virus stocks were aliquoted and stored at −80 °C.

CA p27 quantification and infectivity assay

Quantification of the viral p27 CA protein in purified virus or virus-containing supernatant was done using an SIV p27 Antigen ELISA kit (Zeptometrix Corp.) according to the manufacturer’s instructions. Denaturing gel electrophoresis followed by silver staining of the proteins was also used to quantify p27 based on a comparison to a dilution series of a known amount of a virus stock (SIVmac239/SUPT1-CCR5, obtained from AIDS Vaccine Program, National Cancer Institute at Frederick). Infectivity assays were performed in TZM-BL indicator cells, which contain the luciferase gene and LacZ gene under control of HIV LTR (Wei et al., 2002, and obtained from NIH AIDS Research and Reference Reagent Program). Virus stocks were serially diluted in DMEM medium and used to infect TZM-BL cells (2×10^4 cells per well of a 24-well plate in a volume of 400 μl) in presence of 20 μg/ml DEAE dextran. After a 48-h incubation, the culture medium was removed from each well and the cells were rinsed with PBS. An aliquot of 80 μl of Reporter lysis buffer (Promega Corp.) was added to each well of the 96-well plate for the luciferase assay. After one freeze–thaw cycle, 75 μl of cell lysate was used for measurement of luciferase activity using a luminometer. For X-gal staining, 48 h after infection, cells were fixed in 200 μl of fixing solution (1% formaldehyde, 0.2% glutaraldehyde in PBS) at room temperature for 5 min. The fixing solution was removed and the cells rinsed twice with PBS. Cells were then stained with 200 μl staining buffer (stock is 9.5 ml PBS, 200 μl 0.2 M potassium ferrocyanide, 200 μl of 0.2 M potassium ferricyanide, 10 μl of 2 M MgCl2, 100 μl of 40 mg/ml X-gal stock) at 37 °C for 2 h, washed, and infectious units counted as blue-cell forming units (BUF) using light microscopy.

Protein analysis of virions and cell lysates

Concentrated and purified virions were prepared as described above. Cell lysates were prepared by harvesting the cell monolayer from a 100-mm plate in 2 ml of NP-40 lysis buffer (0.05 M Tris, pH 7.5; 0.1 M NaCl, 0.002 M EDTA; 0.2% NP-40 and 1 protease inhibitor tablet per 25 ml, Roche, Cat# 1873580) 2 days after transfection. Samples were stored at −80 °C until use. For western blot analysis, samples were mixed with gel loading dye, heated at 70 °C for 10 min, then the proteins separated by electrophoresis in a 4–12% SDS–polyacrylamide gel (NuPAGE Novex Bis–Tris Gels, Invitrogen). The proteins were transferred out of the gel onto a Hybond-P PVDF membrane (Amersham Biosciences, NJ, USA) according to standard procedures; the blots were then blocked in 3% membrane blocking agent (Amersham Biosciences, NJ, USA) in PBS with 0.1% Tween, then probed with serum pooled from SIVsm-infected macaques, an SIV Env monoclonal antibody (36D5, a gift from Dr. Jim Hoxie), or an SIVmac251 Gag Monoclonal antibody (KK64, obtained from the NIH AIDS Research and Reference Reagent Program) at a 1:000 dilution. Blots were then incubated with horseradish peroxidase (HRP)-conjugated, anti-human (1:2000) or anti-mouse immunoglobulin G (1:2000) secondary antibodies (Promega, San Luis Obispo CA) and developed with chemiluminescent substrate (Amersham Biosciences, NJ, USA.). Bands were visualized by using Kodak BioMax MR Film.

Expression of labeled viral proteins in cultures infected with either SC27.1 or wild-type virus was analyzed radioimmunoprecipitation. Briefly, infected TZM-BL cells were washed then starved in serum-free, cysteine and methionine-free DMEM medium for 2 h. The cells were labeled overnight by supplementing the medium with 100 μCi/ml S35-Trans Label (ICN Radiochemicals, Costa Mesa, CA). Serum pooled from SIVsm-infected macaques or SIV Env Monoclonal (36D5) or SIVmac251 Gag Monoclonal (KK64) were used to isolate viral proteins. Antibodies were added to cells and virus lysates and incubated overnight at 4 °C with rocking. Protein A and/or Protein G agarose beads were added and the incubation continued for a further 2 h. The beads were washed twice with NP-40 lysis buffer, then boiled in gel loading buffer; the eluted proteins were separated by gel electrophoresis in a 4–12% SDS–polyacrylamide gel. After electrophoresis, the gel was placed on a filter paper and dried in a gel dryer. Dried gels were exposed to X-ray film.

Analysis of SC27.1 Vif function

The activity of the mutant Vif protein in SC27.1 was measured in a single-cycle infectivity assay in presence of APOBEC3G. 293T cells were transfected with SC27.1/pC1 VSV G or wild-type virus, together with pEDNA-APO3G (obtained from the NIH AIDS Research and Reference Reagent Program) at a plasmid molar ratio of 1:10; 1:20; 1:50 and 1:100 (the amount of SC27.1 and wild-type SIVsmH4i was fixed at 2.5 μg per well in a 6-well plate; the SC27.1/pC1 VSV G molar ratio was 1:10). Supernatant virus was harvested at 48 h and used to infect TZM-BL cells as described above. The infectivity titer and p27 titer were determined as described above.

Analysis of Env surface expression

Fragments of SC27.1 or SIVsmH4i env sequence were amplified using PCR and cloned into the VEE replicon expression
Detection of replication competent viruses

Pseudotyped virus generated by transfection of 293T cells with SC27.1 and pCI-G was used to infect 3 × 10⁶ CEM × 174 cells at an MOI of 1. Wild-type virus and SC27.1 virus in the absence of VSV G pseudotype were also used to infect cells. Doses of MOI 1, 0.1, and 0.01 were used for the wild-type virus to infect cells. The infection was done in 48-well plates with a total volume of 250 μl in each well. After incubation for 4 h, cells were washed in PBS and cultured in 25 mm flasks in 10 ml RPMI medium containing 10% FCS. Every other day, the supernatant was collected and half of the cells were removed, the remaining cells were maintained with 10 ml fresh medium. When syncytium formation was observed in the low dose wild-type virus (MOI of 0.01), the supernatant medium from each of the cultures was used to infect fresh CEM × 174 cells (passage 2). Cells were maintained as described above and the passage repeated once more (passage 3). Each passage cultures was maintained for up to 2 weeks. Supernatants collected at various time points were analyzed for p27 level by ELISA.

Acknowledgments

This work was supported by NIH awards PO1-AI46023 and R21 AI064063. We thank Drs. Philip Johnson, Nancy Davis, and Robert Johnston for helpful advice. We also acknowledge the assistance of Dr. Candace Gomez. Numerous essential reagents were obtained from colleagues through the NIH AIDS Research and Reference Reagent Program. Dr. John Rose and Dr. Amiya Banerjee generously provided expression clones of VSV G proteins. This work also benefited from the support of the UNC Center For AIDS Research, funded by NIH award P30 AI050410.

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