

Recombinant rabies virus as potential live-viral vaccines for HIV-1

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Recombinant, replication-competent rabies virus (RV) vaccine strain-based vectors were developed expressing HIV type I (HIV-1) envelope glycoprotein (gp160) from both a laboratory-adapted (CXCR4-tropic) and a primary (dual-tropic) HIV-1 isolate. An additional transcription stop/start unit within the RV genome was used to express HIV-1 gp160 in addition to the other RV proteins. The HIV-1 gp160 protein was stably and functionally expressed, as indicated by fusion of human T cell lines after infection with the recombinant RVs. Inoculation of mice with the recombinant RVs expressing HIV-1 gp160 induced a strong humoral response directed against the HIV-1 envelope protein after a single boost with recombinant HIV-1 gp120 protein. Moreover, high neutralization titers up to 1:800 against HIV-1 could be detected in the mouse sera. These data indicate that a live recombinant RV, a rhabdovirus, expressing HIV-1 gp160 may serve as an effective vector for an HIV-1 vaccine.

rhabdoviruses | RNA | envelope | gp160

Even though great success has been made in the therapy of HIV-1 infection during the last several years (1, 2), the development of a protective HIV-1 vaccine still remains a major goal in halting the HIV-1 pandemic. Most successful vaccines against viral diseases have been composed of killed or attenuated viruses (for review, see ref. 3). This approach seems not to be suitable for HIV-1 because killed HIV-1 virus induces only a poor neutralizing antibody response and no cytotoxic T lymphocyte (CTL) response. The use of attenuated strains of HIV-1 may not be possible based on recent reports indicating that even attenuated simian immunodeficiency virus (SIV) strains can cause an AIDS-like disease in monkeys (4, 5). Nonetheless, the finding that rhesus macaques vaccinated with live, *nef*-deleted SIV were completely protected against challenge with live, pathogenic SIV (6) emphasizes the hypothesis that recombinant viruses are excellent candidates for live vaccines against HIV-1. The most widely used recombinant viral vectors are based on vaccinia virus or canarypox virus expressing HIV-1 genes, mostly the HIV-1 envelope protein, gp160 (7–10). These vectors were able to induce a CTL response and seroconversion in a variety of animals, including humans, but they were unable to induce high-titer neutralizing antibodies against HIV-1 and failed to completely protect chimpanzees from HIV-1 challenge (11).

Rabies virus (RV) is a negative-stranded RNA virus of the rhabdovirus family, and it possesses a relatively simple, modular genome organization coding for five structural proteins (12). The suitability of RV as an expression vector has been recently shown and indicates its potential use as a viral vaccine vector (13, 14). We utilized a RV vaccine strain-based vector, which is nonpathogenic for a wide range of animal species when administered orally or intramuscularly. This approach shows advantages over other viral vectors, for several reasons. First, its modular genome organization makes genetic modification easier than for the majority of more complex genomes of DNA and plus-stranded RNA viruses. Second, large foreign genes that are expressed from the RV genome are highly stable (14). Third,

rhabdoviruses have a cytoplasmic replication cycle, and there is no evidence for recombination and/or integration into the host cell genome (15). In contrast to most other viral vectors, only a negligible seropositivity exists in the human population to RV, and immunization with a RV-based vector against HIV-1 would not interfere with immunity against the vector itself. Because oral immunization against RV with a RV vaccine strain was successful and apathogenic in chimpanzees (J. Cox and U. Wulle, personal communication), a RV-based vector may also be promising in inducing a mucosal immunity against HIV-1. In addition, RV grows to high titers (10^9 foci-forming units) in various cell-lines without killing the cells, which probably results in longer expression of HIV-1 genes compared with a cytopathogenic vector. The results with *nef*-deleted SIV, which protects against lethal SIV challenge, indicates that long term expression of the HIV-1 viral genes may be the key to inducing protection against HIV-1 infection.

We previously reported the use of RV as an expression vector of different reporter genes (16). Here, we test the ability of a RV-based vector to induce an immune response against HIV-1. We cloned the coding region of the HIV-1 gp160 (strains NL4-3 and 89.6) between the RV glycoprotein (G) and polymerase (L) proteins under the control of a RV transcription Stop/Start signal. The resulting recombinant RVs expressed HIV-1 gp160 along with the other RV proteins. HIV-1 envelope protein was stably expressed and elicited a strong humoral immune response in immunized mice after a single recombinant HIV-1 protein boost.

Materials and Methods

Plasmid Construction. Two single sites were introduced in the previously described RV cDNA pSAD L16 (17) upstream of the G (*Sma*I) and Ψ gene (*Nhe*I) by site-directed mutagenesis (GeneEditor, Promega) using the primers RP11 5'-CCTCAA AAGACCCCGGGA AAGATGGTTCCTCAG-3' and RP12 5'-GACTGTAAGGACYGGCTAGCCTT-TCAACGATCCAAG-3', resulting in the plasmid pSN. pSN was the target used to introduce a new transcription Stop/Start sequence, as well as a single *Bsi*WI site using a PCR strategy. First, two fragments were amplified by PCR from pSN using Vent polymerase (New England Biolabs) and the forward primers RP1 5'-TTTTGCTAGCTTATAAAGT-GCTGGGTCATCTAAGC-3' or RP10 5'-CACTACAAGT-CAGTCGAGACTTGG AATGAGATC-3'. The reverse primers were RP18 5'-TCTCGAGTGTCTCTCTCCAACAA-3'

Abbreviations: CTL, cytotoxic T lymphocyte; SIV, simian immunodeficiency virus; RV, rabies virus; moi, multiplicity of infection; VSV, vesicular stomatitis virus.

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and RP17 5'-AAGCTAGCAAAAACGTACGGGAGGGGT-GTTAGTTTTTTTCATGGAAGTGGATCGTTGAAAG-GACG-3'. RP17 contains a RV transcription Stop/Start sequence (underlined) and a *Bsi*WI and *Nhe*I site (italicized). PCR products were digested with *Nhe*I and were ligated, and the 3.5-kb band was eluted from an agarose gel. After gel elution, the band was digested with *Cl*aI/*Mlu*I and was ligated to the previously *Cl*aI/*Mlu*I-digested pSN. The plasmid was designated pSBN.

The HIV-1 gp160 genes, encoding the envelope protein of the HIV-1 strains 89.6 and NL4-3, were amplified by PCR using *Vent* polymerase, the forward primer 5'-GGGCTG-CAGCTCGAGCGTACGAAAATGAGAGTGAAGGA-GATCAGG-3' containing *Pst*I/*Xho*I/*Bsi*WI sites (italicized), and the reverse primer 5'-CCTCTAGATTATAGCAAAGC-CCTTCCAAG-3' containing a *Xba*I (italicized) site. The PCR products were digested with *Pst*I and *Xba*I and were cloned to pBluescript II SK(+) (Stratagene). After conformation of the sequence, the HIV-1 gp160 genes were excised with *Bsi*WI and *Xba*I and were ligated to pSBN, which had been digested with *Bsi*WI and *Nhe*I. The resulting plasmids were entitled pSBN-89.6 and pSBN-NL4-3.

Recovery of Infectious RV from cDNA. For rescue experiments of the recombinant RVs, we used the previously described vaccinia virus-free RV recovery system (18). In brief, BSR-T7 cells (19), which stably express T7 RNA polymerase (a generous gift of S. Finke and K.-K. Conzelmann, Genzentrum, Munich) were transfected with 5 μ g of full-length RV cDNA in addition to plasmids coding for the RV N-, P-, and L-proteins (2.5, 1.25, and 1.25 μ g, respectively), using a Ca₂PO₄ transfection kit (Stratagene) as indicated by the vendor. Three days after transfection, tissue culture supernatants were transferred onto fresh BSR cells, and infectious RV was detected three days later by immunostaining against RV the N protein (Centocor).

One-Step Growth Curve. BSR cells (a BHK-21 clone) were plated in 60-mm dishes and 16 hours later were infected (7×10^6 cells) with a multiplicity of infection (moi) of 10 with SBN, SBN-89.6, or SBN-NL4-3 in a total volume of 2 ml. After incubation at 37°C for 1 hour, inocula were removed and cells were washed four times with PBS to remove any unabsorbed virus. Three milliliters of complete medium was added back, and 100 μ l of tissue culture supernatants were removed at 4, 16, 24, and 48 hours after infection. Virus aliquots were titered in duplicate on BSR cells.

Immunization. Groups of five 4- to 6-week-old female BALB/c mice obtained from The Jackson Laboratory were inoculated subcutaneously in both rear footpads with 10^6 foci-forming units of SBN, SBN-89.6, or 10^5 NL4-3 in DMEM + 10% FBS. Three of five mice in each group were boost-immunized intraperitoneally 3 months after infection with 10 μ g of recombinant gp41 (IIIB, Intracel, Issaquah, WA) and 10 μ g of recombinant gp120 (IIIB, Intracel) in 100 μ l of complete Freund's adjuvant.

Enzyme-Linked Immunosorbent Assay (ELISA). Recombinant HIV-1 gp120 (IIIB strain, Intracel) was resuspended in coating buffer (50 mM Na₂CO₃, pH 9.6) at a concentration of 200 ng/ml and was plated in 96-well ELISA MaxiSorp plates (Nunc) at 100 μ l in each well. After overnight incubation at 4°C, plates were washed three times (PBS, pH 7.4/0.1% Tween-20), were blocked with blocking buffer (PBS, pH 7.4/5% dry milk powder) for 30 minutes at room temperature, and were incubated with serial dilutions of sera for 1 hour. Plates were washed three times, followed by the addition of horseradish peroxidase-conjugated goat anti-mouse-IgG (H+L) secondary antibody (1:5,000, Jackson ImmunoResearch). After a 30-minute incubation at 37°C, plates were washed three times, and 200 μ l of OPD-substrate

(*o*-phenylenediamine dihydrochloride, Sigma) was added to each well. The reaction was stopped by the addition of 50 μ l of 3 M H₂SO₄ per well. Optical density was determined at 490 nm.

Western Blotting. Human T-lymphocytic cells (Sup-T1) cells were infected with a moi of 2 for 24 hours and were resuspended in lysis buffer [50 mM Tris, pH 7.4/150 mM NaCl/1% Nonidet P-40/0.1% SDS/1 \times protease inhibitor mixture (Sigma)] for 5 minutes. The protein suspension was transferred to a microfuge tube and was spun for 1 minute at 10,000 \times g to remove cell debris. Proteins were separated by 10% SDS/PAGE and were transferred to a PVDF-Plus membrane (Osmonics, Minnetonka, MN). After blocking for 1 hour [5% dry milk powder in PBS (pH 7.4)], blots were incubated with sheep α -gp120 antibody (AR-RRP) (1:1,000) or human α -rabies sera (1:500) in blocking buffer for 1 hour. Secondary antibodies of goat α -human or donkey α -sheep horseradish peroxidase-conjugated antibodies (1:5,000) (Jackson ImmunoResearch) were added, and blots were incubated for 1 hour. Each antibody incubation was followed by three washes with WB-wash buffer (PBS, pH 7.4/0.1% Tween-20). Chemiluminescence (NEN) was performed as directed by the manufacturer.

Western blot analysis to detect anti-HIV-1 antibody was performed by using a commercial Western Blot kit (QualiCode HIV-1/2 Kit, Immunetics, Cambridge, MA) according to the manufacturer's instructions, except for the mouse sera in which α -human IgG conjugate was substituted with a 1:5,000 dilution of an alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch).

Virus Neutralization Assays. HIV-1 strains were recovered on 293T cells. Virus stocks were expanded on MT-2 cells (HIV-1 NL4-3), frozen at -75°C, and titered on MT-2 cells. Neutralization assays were performed according to Montefiori *et al.* (20). In brief, $\approx 5,000$ TCID₅₀ of HIV-1_{NL4-3} were incubated with serial dilutions of mouse sera for 1 hour. MT-2 cells were added and incubated at 37°C, 5% CO₂ for 4-5 days. Cells (100 μ l) were transferred to a poly-L-lysine plate and were stained with neutral red dye (Neutral Red, ICN) for 75 minutes. Cells were washed with PBS, were lysed with acid alcohol, and were analyzed by using a colorimeter at 550 nm. Protection was estimated to be at least 50% virus inhibition.

Results

Construction of Recombinant RVs Expressing HIV-1 Envelope Protein.

To generate RV recombinant viruses expressing HIV-1 gp160, we constructed a new vector based on the previously described infectious RV cDNA clone pSAD-L16 (13). By using site-directed mutagenesis and a PCR strategy, the ψ gene was deleted from the RV genome, and a new transcription unit, containing a RV Stop/Start signal and two single sites (*Bsi*WI and *Nhe*I), was introduced into the RV genome. The resulting plasmid was designated pSBN (Fig. 1). SBN was recovered by standard methods and displayed the same growth characteristics and similar viral titers as SAD-L16, indicating that neither the deletion of the Ψ gene nor the new transcription unit affected the RV vector (data not shown). The HIV-1 envelope genes (NL4-3 and 89.6) to be expressed from SBN were generated by PCR and were cloned between the *Bsi*WI and *Nhe*I sites, resulting in the plasmids pSBN-NL4-3 and pSBN-89.6 (Fig. 1). All constructs were checked via DNA sequencing.

Recombinant RVs expressing either HIV-1_{NL4-3} or HIV-1_{89.6} envelope proteins were recovered by transfection of cells stably expressing the T7-RNA-polymerase with plasmids encoding the RV N, P, and L proteins along with a plasmid coding for the respective RV full-length antigenomic RNA. Three days after transfection, supernatants of transfected cells were transferred to fresh cells and three days later were analyzed by indirect

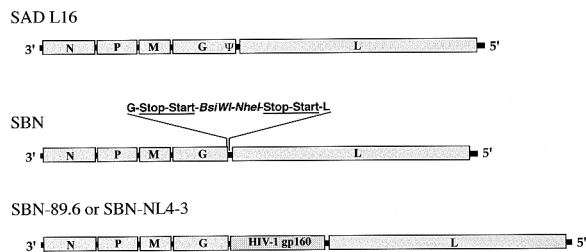


Fig. 1. Construction of recombinant RV genomes. At the top, the wild-type RV genome with its five ORFs is shown (SAD L16). By using a PCR strategy and site-directed mutagenesis, the entire Ψ gene was removed, and a new minimal RV transcription unit containing two single sites was introduced between the G and L genes (SBN). The cDNA sequence encoding HIV-1_{89.6} or HIV-1_{NL4-3} gp160 was inserted by using the *Bs*WI and *Nhe*I sites resulting in the plasmids pSBN-89.6 or pSBN-NL4-3 (bottom).

immunofluorescence microscopy for expression of HIV-1 gp160. A positive signal for gp160 in cells infected with recombinant SBN-NL4-3 and SBN-89.6 confirmed the successful recovery of recombinant RVs expressing HIV-1 envelope protein (data not shown).

Growth Characteristics of Recombinant RVs. A 3-fold lower titer for SBN-NL4-3 and a 10-fold titer reduction for SBN-89.6 was noticed, as compared with wild-type SBN. To examine the differences in virus replication in detail, a one-step growth curve of the recombinant RVs was performed. BSR cells were infected with a moi of 10 to allow synchronous infection of all cells. After replacing the virus inoculum with fresh medium, viral titers were determined at the indicated time-points (Fig. 2). Both recombinant RVs expressing HIV-1 gp160 replicated at only a slightly reduced rate compared with wild-type RV, with the final titers being 2.3- (SBN-NL4-3) or 8-fold (SBN-89.6) reduced. The 20% longer genome size of the recombinant RVs cannot explain the slower growth of these viruses. A recombinant RV expressing a 1.9-kb gene (firefly luciferase) grew to wild-type RV titers (14). It was previously shown for another rhabdovirus, vesicular stomatitis virus (VSV), that certain glycoproteins expressed by VSV, such as the HIV-1 envelope protein, reduces the titer of these viruses whereas others do not (21–23).

Expression of HIV-1 gp160 by Recombinant RVs. To ensure the expression of HIV-1 gp160 by the recombinant viruses, cell lysates from recombinant RV infected cells were analyzed by Western immunoblotting with an antibody directed against RV (Fig. 3 *Left*) or HIV-1 gp120 (Fig. 3 *Right*). Two bands of the expected size for HIV-1 gp160 and gp120 were detected in lysates from cells infected with SBN-89.6 or SBN-NL4-3 (Fig. 3

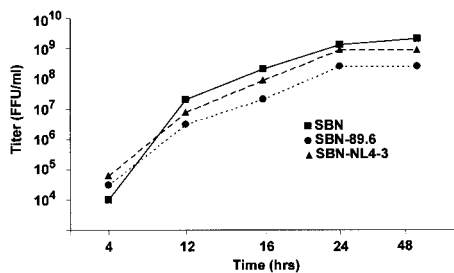


Fig. 2. One-step growth curves of recombinant RV. BSR cells were infected with the recombinant RVs (SBN, SBN-89.6, and SBN-NL4-3), as described in *Materials and Methods*, and viral titers were determined in duplicate at the indicated time-points.

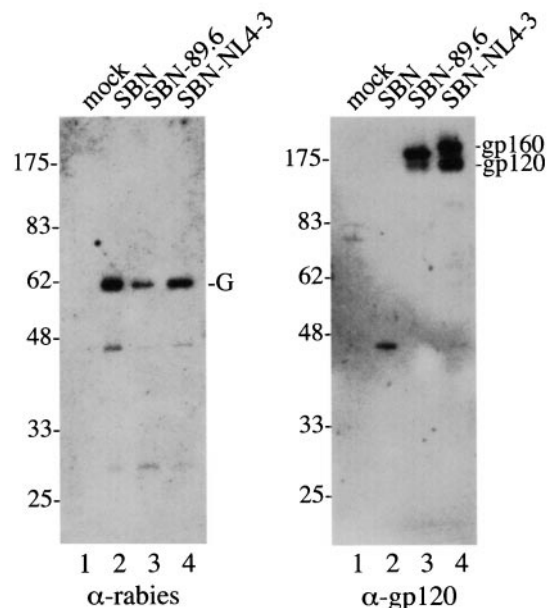


Fig. 3. Western blot analysis of recombinant RVs expressing HIV-1 gp160. Sup-T1 cells were infected with a moi of 2 with SBN, SBN-89.6, or SBN-NL4-3 and were lysed 24 hours later. Proteins were separated by SDS/PAGE and were analyzed by Western blotting. An antibody directed against gp120 detected two bands at the expected size for HIV-1 gp160 and gp120 in cell-lysates infected with SBN-89.6 or SBN-NL4-3 (*Right*, lanes 3 and 4). No signal was detected either in the mock or SBN-infected cells (*Right*, lanes 1 and 2). Successful infection of the cells by the recombinant RVs was confirmed with a polyclonal antibody directed against RV (*Left*, lanes 2, 3, and 4).

Right, lanes 3 and 4) but could not be observed in cell lysates of mock-infected or SBN infected cells (Fig. 3, *Right*, lanes 1 and 2). The Western blot probed with an α -RV antibody confirmed that all viruses (Fig. 3 *Left*, lanes 2, 3, and 4) infected the target cells.

To determine whether the HIV-1 envelope protein is functionally expressed from RV, we analyzed the recombinant RVs in a fusion assay in a human T cell-line (Sup-T1). Preliminary results confirmed that wild-type RV is able to infect and replicate in human T cell-lines. Because wild-type RV infects cells by receptor-mediated endocytosis, the RV glycoprotein (G) can only cause fusion of infected cells at a low pH (24). In contrast to wild-type RV, large syncytium-formation was detected in Sup-T1 cells 24 hours after infection with SBN-89.6 or SBN-NL4-3 (Fig. 4). These results indicated that the expressed HIV-1 envelope proteins were properly folded and transported to the cell surface and could be recognized by the HIV-1 receptor and coreceptor, CD4 and CXCR4.

Envelope protein from the dual-tropic HIV-1 strain (89.6) should be able to induce cell fusion if coexpressed with CD4 and CCR5 whereas NL4-3 gp160 should only induce fusion on cells expressing CD4 and the HIV-1 coreceptor CXCR4. Infection of 3T3 murine cells expressing human CD4 did not result in cell fusion regardless of the recombinant RV used whereas syncytium-formation was detected in 3T3 cells expressing CD4 and CXCR4 after infection with SBN-NL4-3 or SBN-89.6. As expected, only expression of HIV-1_{89.6} envelope protein in 3T3 cells, expressing CD4 and CCR5, caused fusion of these cells (data not shown).

Anti-gp120 Antibody Response in Mice Infected with RV Expressing HIV-1 gp160. One likely requirement for a successful HIV-1 vaccine would be the ability to induce a strong humoral response against the HIV-1 protein gp160. To determine whether the recombinant gp160 proteins expressed by recombinant RV were

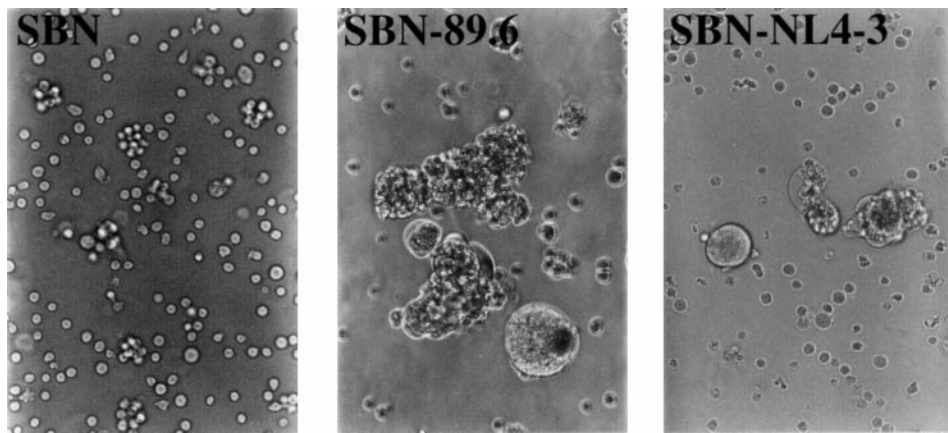


Fig. 4. Sup-T1 cells were infected by using a moi of 1 with SBN, SBN-89.6, or SBN-NL4-3. Twenty-four hours after infection, syncytia-formation was detected in cell cultures infected with recombinant RV expressing HIV-1 gp160 (Center and Right), indicating expression of functional HIV-1 envelope protein. No cell fusion was detected in cultures infected with wild-type RV (Left).

able to induce an anti-HIV-1 immune response, groups of five BALB/c mice were inoculated subcutaneously in both rear footpads with 10^6 foci-forming units of SBN, SBN-89.6, or 10^5 foci-forming units SBN-NL4-3. Mice were bled 11, 24, and 90 days after the initial infection with RV, and the sera were analyzed by ELISA. No response to the HIV-1 envelope was detected in the sera of immunized animals, but an ELISA using RV glycoprotein, instead of HIV-1 gp120, as an antigen confirmed the RV infection and detected high level of antibodies against RV as early as 11 days after infection. Several studies on viral vectors expressing HIV-1 gp160 indicated that a booster infection or a boost with a recombinant protein is necessary to induce detectable serum antibody response against HIV-1 envelope protein. The high antibody titer detected in the RV ELISA indicated that an additional infection with the recombinant RV would not be promising; therefore, we boosted three of five mice from every group with $10 \mu\text{g}$ of recombinant gp120 and gp41 in complete Freund's adjuvant. Twelve days after the subunit boost, the mice were bled, and the immune response was analyzed by an HIV-1 gp120 ELISA. The results demonstrated that an HIV-envelope subunit boost elicited a strong immune response against HIV-1 gp120 only in mice previously infected with SBN-89.6 or SBN-NL4-3 (Fig. 5). Wild-type RV (SBN)-infected mice reacted only in the lowest serum dilution (1:160) after the boost. An ELISA specific for HIV-1 gp41 was negative for all mouse sera, even after the boost with recombinant HIV-1 gp120/gp41.

These data were also confirmed by Western blot analysis (Fig. 6). Only sera from mice infected with SBN-89.6 or SBN-NL4-3 and subsequently boosted with recombinant protein were able to react with gp120 whereas all other sera failed to detect any HIV-1 protein. None of the sera had gp41-specific bands, even with a gp41 subunit immunization.

Primary Virus Infection Followed by Recombinant Protein Boost Induces Neutralizing Antibodies Against HIV-1. HIV-1 neutralizing antibody (NA) titers were determined in MT-2 cells by a vital dye staining assay using HIV-1_{NL4-3}. The mouse serum was able to neutralize a tissue culture laboratory-adapted HIV-1_{NL4-3} strain at a 1:800 serum dilution after immunization with SBN-NL4-3 and an envelope subunit booster injection of recombinant gp120 (IIBB strain) whereas immunization with SBN-NL4-3 did not induce detectable neutralizing antibody. These results were confirmed in two independent experiments. The sera from wild-type RV (SBN)-infected mice that received a recombinant gp120 boost displayed only a very low neutralizing antibody titer

of 1:50 (Table 1). These results indicate that a boost injection with recombinant gp120 after the priming with recombinant RV expressing HIV-1 gp160 is essential to elicit high titers of neutralizing antibody.

Discussion

The data presented herein demonstrate that a recombinant RV expressing a full-length HIV-1 envelope protein can be generated. The foreign gene was stably expressed by replication competent RV and induced a strong humoral response in mice against HIV-1_{NL4-3} envelope protein after infection with recombinant RV and a single subsequent boost of HIV-1 gp120 protein. The ability of the murine sera to neutralize HIV-1 strain NL4-3 was demonstrated.

A requirement for blocking an HIV-1 infection is that antibodies must bind to the viral surface glycoprotein. Unfortunately, the HIV-1 virion-associated trimer gp41/gp120 seems to be the less immunogenic form of the glycoprotein. In addition,

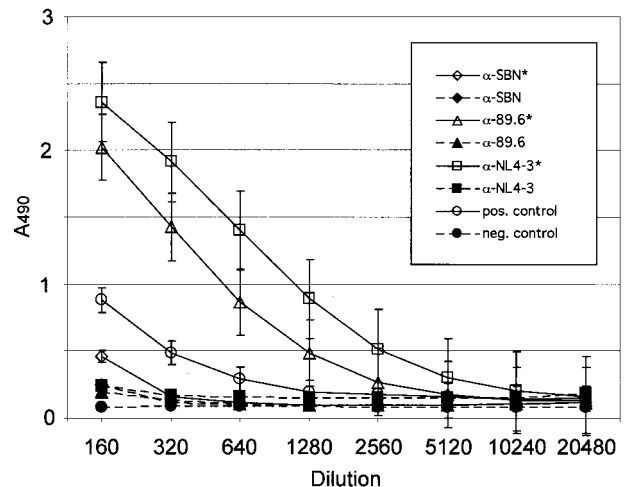


Fig. 5. ELISA reactivity of mouse sera against HIV-1 gp120. Five mice each were immunized with recombinant RVs (SBN, SBN-89.6, or SBN-NL4-3), and, 3 months after the initial infection, three mice of each group were boosted with recombinant HIV-1 gp120 and gp41 (SBN*, SBN-89.6*, or SBN-NL4-3*). Each data point on the graph indicates the average of mice from each group in three independent experiments. One mouse of the SBN-89.6 group did not react to the boost injection and was not included in the graph. The error bars indicate the standard deviations.

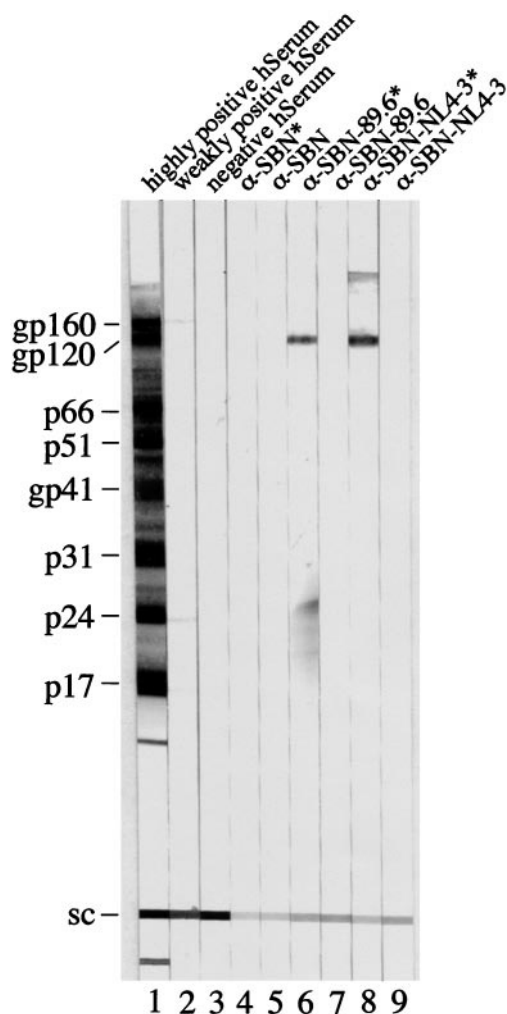


Fig. 6. Western blot analysis of mice serum antibody response to HIV-1 antigens. Sera from one mouse of each group (SBN, SBN-89.6, or SBN-NL4-3), which were immunized by the RVs (α -SBN, α -SBN-89.6, or α -SBN-NL4-3) or were immunized and boost-injected with recombinant gp120 and gp41 (α -SBN*, α -SBN-89.6*, or α -SBN-NL4-3*), were tested at 1:100 dilutions by clinical Western blot as described in *Materials and Methods*. A highly positive and weakly positive human control serum was used to detect the position of the HIV-1 proteins. SC indicates the serum control.

there is evidence that, in a natural HIV-1 infection, the humoral response is mainly directed to viral debris, i.e., unprocessed HIV envelope protein, and not against the virus itself (25). A primary strong reaction against epitopes not exposed on the mature HIV-1 gp41/gp120 may hamper the later response against the epitopes in the mature HIV-1 envelope protein, which are the targets for neutralization antibodies. This may explain why

Table 1. HIV-1 neutralizing antibody titer

Immunization with boost injection of recombinant HIV-1 gp120/gp41	HIV-1 _{NL4-3} neutralizing antibody titer	
	Experiment 1	Experiment 2
SBN-NL4-3	<1:50	1:50
SBN-NL4-3*	1:800	1:800
SBN*	<1:50	<1:50

*Boost injection with recombinant gp120 after priming with SBN or SBN-NL4-3.

cytopathogenic viral vectors, such as vaccinia or avipox, induce only poor neutralizing antibodies (26). Nevertheless, the finding that rhesus monkeys vaccinated with live SIV deleted in *nef* were completely protected against challenge with live, pathogenic SIV suggests that recombinant viruses are excellent candidates for live vaccines against HIV-1 (27). In the case of a RV-based vector, there exists a strong possibility that the induction of mature oligomers of HIV-1 envelope proteins may occur, which may be required for an induction of a strong immune response (28–30). Misfolded proteins are retained and degraded within the cell (31) and, in the case of a RV-based vector, they would not be released, as opposed to a vector, which induces cytopathogenesis. RV infects most mammalian cells but causes only a very mild cytopathogenic effect in certain cell-lines, such as BHK-21 S13 and chick embryo fibroblast (32, 33). One safety concern could be that a RV-based vector expressing HIV-1 gp160 will cause fusion of human T cells, as shown in Fig. 4, but this process may be even helpful by exposing HIV-1 gp160 epitopes that are normally not seen by the immune system.

As with other viral vectors expressing HIV-1 gp160, we were not able to detect a humoral response against gp120 after the initial priming with the recombinant RVs, but a strong response after a boost with recombinant HIV-1 gp120 and gp41. There was no response to HIV-1 gp41 by clinical Western blot or an HIV-1 gp41 ELISA, probably because of degradation of the recombinant gp41 used in these initial studies.

The sera of the SBN-NL4-3 primed mice were able to neutralize HIV-1_{NL4-3}, and further experiments will analyze whether HIV-1 gp160 expressed by RV vectors induces antibodies against more conserved epitopes between various HIV-1 strains and, therefore, are able to induce cross-neutralization against different HIV-1 strains. Different polyclonal sera that cross-neutralize a large number of different HIV-1 strains are often directed against the CD4 binding domain (34). Further experiments will analyze whether this is also the case for antibodies induced by RV-based vectors. We detected a low neutralizing antibody response (1:50) against HIV-1 with sera from mice immunized with wild-type RV and boosted with recombinant HIV-1 gp120. The reason for this is not clear, but a recent report indicates there may be some low-level cross-reacting antibodies between the RV glycoprotein and HIV-1 gp120 (35).

In addition to a boost of recombinant protein, an infection with a recombinant virus expressing gp160 from the same or a different HIV-1 strain may induce an even stronger response, especially against conformational-dependent epitopes. It was previously shown for adenovirus that boost infection with a heterotypic adenovirus expressing HIV-1 envelope protein significantly enhanced the humoral response against HIV-1 gp160 (36). We are currently constructing recombinant RVs expressing HIV-1 gp160, where the RV glycoprotein (G) is replaced with that of VSV G (serotype Indiana or New Jersey). Because rhabdoviruses have only a single surface protein on their virions, chimeric RV/VSV viruses should not be neutralized by the humoral response against the RV G and therefore allow a second productive infection. The use of a recombinant chimeric RV/VSV would have an advantage, compared with the use of recombinant HIV-1 gp120, to display the properly folded HIV-1 envelope protein on the surface of the infected cell. In addition, repeated expression of the RV nucleoprotein, which was previously shown to be an exogenous superantigen (37, 38), might help to enhance the immune response against the HIV-1 envelope. The RV N protein is an excellent immunogen and was shown to enhance the immune response against feline immunodeficiency virus, when used in combination with the feline immunodeficiency virus surface glycoproteins (39).

The goal of this study was to examine whether a RV-based vector is able to induce an immune response against HIV-1 gp160. We used a vaccine strain-based RV vector, which is

nonpathogenic for a wide range of animal species when administered orally or intramuscularly. Live-attenuated RV is currently not used for immunization of humans, but preliminary data showed that the recombinant RVs expressing HIV-1 envelope protein are even more attenuated than the vector itself. This, in addition to a RV glycoprotein from a low virulence strain such as RV-SAG₂, may result in a RV-based vector which is safe for use in humans.

This report describes the use of a nonsegmented negative-stranded RNA virus expressing HIV-1 genes as an HIV-1 vaccine. Studies involving another member of the rhabdovirus family, namely VSV, showed that these vectors are able to induce a protective immune response against influenza virus (40), but it still remains to be shown whether the same approach works for the previously constructed VSV expressing HIV-1 gp160 (22). Of note, a recombinant replication-deficient vaccinia virus expressing influenza virus hemagglutinin and nucleoprotein genes protect mice from influenza challenge (41), but recombinant vaccinia virus expressing HIV-1 gp160 failed to protect chimpanzees against HIV-1 (11).

Our results indicated that recombinant RVs are excellent vectors for B cell priming. RV also can induce strong CTL responses (42,

43). Preliminary data demonstrated that the recombinant RV expressing HIV-1 envelope protein induces a specific CTL response against HIV-1_{NL4-3} gp160. Further experiments will analyze whether similar B cell and CTL responses against expressed foreign proteins, such as HIV-1 gp160 or Gag, are possible and will analyze whether recombinant RVs expressing multiple HIV-1 antigens are even more potent live-viral vectors.

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