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INTRODUCING A FRAMESHIFT MUTATION TO THE *POL* SEQUENCE OF HIV-1 PROVIRUS AND EVALUATION OF THE IMMUNOGENIC CHARACTERISTICS OF THE MUTATED VIRIONS (RINNL4-3)

© 2012 Rezvan Zabihollahi[1, 2]*, Seyed Mehdi Sadat[1], Rouhollah Vahabpour[1], Mansoor Salehi[3], Kayhan Azadmanesh[3], Seyed Davar Siadat[1], Ali Reza Azizi Saraji[1, 4], Mohamamd Hassan Pouriavali[1], Seyed Bahman Momen[1]and Mohamad Reza Aghasadeghi[1]**

[1]*Hepatitis and AIDS Department, Pasteur Institute of Iran, Tehran, Iran*

[2]*Isfahan University of Medical Sciences IUMS, Isfahan, Iran*

[3]*Virology Department, Pasteur Institute of Iran, Tehran, Iran*

[4]*Scientific Society of Veterinary Science, Science and Research Branch, Department of Veterinary Laboratory of Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran*

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Inactivation of the reverse transcriptase (RT) and integrase (IN) enzymes can abolish the replication of the human immunodeficiency virus (HIV) and, thus, its infectivity. Here, inactivated HIV particles convenient for designing virus-like particle (VLP) based vaccines have been produced. Inactivated HIV-provirus was created by introducing a frame shift mutation. HIV provirus DNA was cut in the *pol* region by Age I restriction enzyme, followed by filling of sticky ends using the Klenow fragment before ligation. The resulting plasmid was named as pRINNL4-3. HEK-293T cells were used as producer, after being transfected with the modified plasmid. Viral particle production and biological activity were assayed by virus capsid protein (p24) quantification and syncytium formation in MT2 cells, respectively. The immunogenicity of the RINNL4-3 virions was investigated in a mouse model. The mutation was expected to inactivate the virus RT and IN enzymes. The results showed that the VLPs were assembled, as measured by the p24 load of the culture supernatant, and contained functional envelope proteins (Env) as monitored by the syncytium formation. However, these VLPs had no ability to infect target MT2 cells, as well as their VSVG (vesicular stomatitis virus-glycoprotein) pseudotyped counterparts infected HEK-293T cells. A high level of antibody response was observed in immunized mice. Since RINNL4-3 virions are replication incompetent, they are convenient for production and use in biomedical studies. Also, RINNL4-3 is a candidate for a vaccine development due to it contains envelope and structural virus proteins which are crucial for triggering neutralizing antibodies and the cellular immune response.

***Keywords*: HIV-1, reverse transcriptase, integrase, AIDS vaccine.**

The human immunodeficiency virus (HIV) is an RNA-containing virus that belongs to the family of *Retroviridae*. A HIV transcribes its RNA genome to DNA using own reverse transcriptase (RT), and then the proviral DNA is incorporated into the host cell's genome by the virus integrase (IN) enzyme. Infection with HIV causes acquired immunodeficiency syndrome (AIDS) in humans, which is a chronic immune deficiency illness that leads to a severe decrease in the CD4 lymphocyte count.

Cellular and humoral immune responses to HIV virions play a critical role during the virus replication [1, 2]. A strong cellular immune response (CD8-response) to the HIV antigens begins during first weeks of infection [1]. The initial cellular response to HIV antigens correlates with a decline in the viral load after the first viremia [3]. The humoral immune response to HIV infection consists of neutralizing antibodies which are able to neutralize HIV virions [4]. The major target of the neutralizing antibodies is surface glycoprotein (Env) of the HIV which is composed of gp41 and gp120 subunits [4, 5]. Recombinant Env is not effective in trigger the production of neutralizing antibodies [5]. Results of phase III clinical trials showed that vaccination with recombinant gp120 could not protect or even influence the progress of the disease in HIV-infected people [6]. The inability of recombinant HIV surface antigens to broadly trigger the neutralizing antibodies may be due to the differences in the structure of Env on the surface of HIV particles and the recombinant particle-free Env units [5]. In previous studies it was shown that the Env units that are only a part of lipid envelope of virus-like particles (VLPs) can trigger all of the neutralizing antibodies against the HIV Env antigen [7, 8]. Inactive HIV particles are suitable for vaccine investigations because

* E-mail: Rezvan_z_m@yahoo.com; ** mrasadeghi@pasteur.ac.ir

they contain all the immunogenic structural units, as well as the surface antigens in their native forms [5, 9]. It was shown that inactive and attenuated virions can elicite protective humoral and cellular immune responses [10, 11].

HIV virions contain two RNA copies [12]. After fusion of the viral envelope to the cell membrane the pre-integrating complex (PIC) is released into the cytoplasm of target cells. The PIC includes the proviral RNA, IN and RT as its major parts. The function of RT is to synthesize proviral dsDNA that is then integrated into the host's DNA by IN [13]. The transcription and translation systems of the cell recognize the integrated proviral DNA and produce the viral mRNAs and then proteins.

As it was shown earlier, on the first hand, inactivation of RT and IN enzymes blocked the replication of HIV; even a single amino acid substitution in the enzyme catalytic domain leading to abolishing its function [14, 15]. On the other hand, a non-covalent interaction between the virus Gag-polyprotein and proviral RNA triggers packaging and, consequently, assembling of the viral particles [16, 17]. Here, a frame shift mutation was introduced to *pol* sequence of HIV provirus to block the activity of RT and IN without interfering in packaging and assembling processes. The production, biological function and immunological properties of the new mutant HIV-1 molecular clone (pRINNL4-3), which carries inactivated RT and IN enzymes, are also evaluated in this study.

EXPERIMENTAL

Plasmids and the site directed mutagenesis. pNL4-3, a recombinant provirus clone, which contains DNA from the HIV-1 NY5 and LAV strains in the pUC18 vector was used [18] (plasmid was obtained from National Institutes of Health, USA). pMD2.G, pLOX-CWgfp and psPAX2 (www.addgene.org) were used for preparing of VSVG (vesicular stomatitis virus-glycoprotein) pseudotyped and single-cycle replicable (SCR) virions. The Age I (Bsht1) restriction enzyme ("Fermentas", Lithuania) was used to introduce the frame shift mutation. The Age I recognises and cleaves the ACCGGT sequences. This restriction enzyme cuts the pNL4-3 vector only in one site at the 3485 position. Two sticky ends of the digested pNL4-3 were filled using the Klenow fragment enzyme ("Fermentas"). Blunt ends were ligated using the T4 DNA-ligase ("Fermentas"). To confirm the frame shift mutation, pRINNL4-3 was digested with AgeI and run on a 0.7% agarose gel. The size of the mutated plasmid was assessed after linearization with BamHI ("Fermentas").

Cell culture and virus production. Cell lines were maintained as describe previously [19] in RPMI 1640 or DMEM ("Chemicon", USA) supplemented with 15% fetal bovine serum ("Gibco", USA), 2 mM glutamine, 100 U/mL of penicillin G, 100 mg/mL of streptomycin, and 25 mM HEPES. HEK-293T cells were used for virion production. For the transfection of HEK-293T cells the polyfect reagent ("Qiagen", Germany) was used. HEK-93T cells were placed onto 24-well plates at 7×10^4 cells per well, in one day before the transfection. The culture medium was replaced with 300 μL of fresh medium 3 h before the transfection. The transfection was done by mixing 400 ng of DNA and 4 μL of polyfect (for each well), following the protocol provided by the manufacturer. The virus-containing supernatant was harvested at 36, 48 and 72 h [20] and clarified by centrifuging at $6000 \times g$ for 10 min. The virions were pelleted by centrifuging at $50000 \times g$ for 2 h at 4°C and then resuspended by gentle vortexing in DMEM overnight. Virus stocks were analysed for the p24 load (HIV p24 ELISA kit; "Cell Biolabs", USA) and then stored at −70°C. VSVG pseudotyped SCR virions were prepared by cotransfection of HEK-293T cells with psPAX2, pRINNL4-3 and pMD2*G* [20]. These virions were used to infect HEK-293T cells. Green fluorescence protein (GFP) reporter HIV virions were produced by psPAX2 and pLOX-CWgfp, and the RINNL4-3 viral particles were constructed by transfecting the pRINNL4-3 into the HEK-293T cells.

Replication and syncytium formation assays. MT-2 and HEK-293T cells were used as the host of HIV infection. The cells were placed onto 24-well plates at 8×10^4 cells per well in 200 μL of fresh medium and infected with HIV (600 ng of p24). After 5 h, the cells were washed three times with phosphate buffered saline (PBS) and resuspended in 500 μL of the fresh medium. The HEK-293T cell supernatant was harvested after 72 h. The MT-2 cell supernatant was harvested every three days or 24 h (depending on the test). Cell supernatants were assessed by quantitative p24 ELISA kit (HIV p24 ELISA, "Cell Biolabs"). Syncytium formation was quantified by counting the number of syncytiums in five fields by visual inspection after infecting the cells with HIV (6 μg of p24) as described before [19].

Mice immunization against VLPs of HIV. Five female BALB/c mice aged 6–8 weeks were given 40 μg of purified VLP in complete Freund's adjuvant subcutaneously (s.c.). Three weeks later, the same dose of antigen in incomplete Freund's adjuvant was injected s.c. as a booster. Blood samples were obtained from the retro-orbital sinuses of mice at weeks 0, 3, 6 and stored at −20°C prior to analysis. The antibody response to the HIV Env was evaluated using a quantitative ELISA kit ("Biomerieux", France).

RESULTS

Mutation

In this study we introduced a mutation which inactivates RT and IN enzymes. This mutation shifts the reading frame (ORF) of the gag-pol mRNA in the

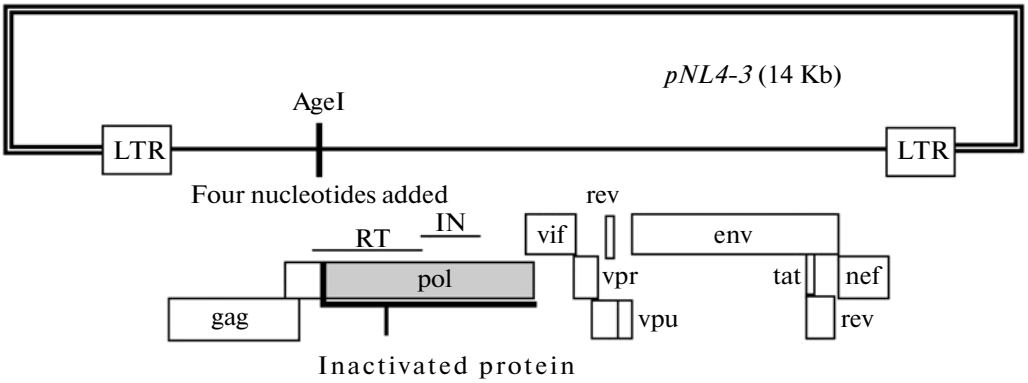

Fig. 1. The restriction site of the Age I restriction enzyme (nucleotide site 3485) is shown in the HIV-1 provirus (pNL4-3). Cutting the plasmid at this site and ligating the ends after filling them results in addition of four nucleotides to the Age I site. The ORF of the gag-pol mRNA is changed downstream of the Age I restriction site. Almost 44% of RT and the entire IN proteins are inactivated by this way.

polymerase (*pol*) region by which the pol-part of the gag-pol polyprotein is coded (fig. 1). The ORF-shift mutation changes the 248 codon at the 3'-end of the RT mRNA (the whole RT sequence is 560 codons) and also completely changes the ORF within mRNA for IN. The mutation was verified by separation of the fragments resulting from the digestion of pNL4-3 and pRINNL4-3 with Age I in an agarose gel. The pNL4-3 was completely digested by Age I, while the pRINNL4-3 did not (fig. 2). One 14-kb band which correlates with the linear pNL4-3 can be seen in fig. 2.

Production of mutated virions

Cells were transiently transfected with RINNL4-3. Forty eight hours later the virions were harvested and analysed for the viral capsid protein load using a quantitative p24 ELISA kit. The load of p24 in the supernatant of cells producing RINNL4-3 virions was 830 ng/mL that was similar to the control. Viral particles produced from psPAX2 and pLOX-CWgfp transfected cells were considered as the control and the p24 load of 1200 ng/mL was detected in the supernatant from these cells.

Infectivity of the RINNL4-3 virions

Infectivity of the mutated virions (RINNL4-3) was checked using two target cells, MT-2 and HEK-293T. The MT-2 cell line is competent to be infected with HIV via the HIV surface glycoprotein (Env). No reproduction of mutated virions in MT-2 cells was detected even after a 21 day follow up. SCR HIV virions were considered as a positive control of the replication, and measurable increase in the p24 load of the SCR HIV infected cells was detected at day 3 after the infection.

Infectivity of the VSVG pseudotyped RINNL4-3 virions was also assessed in HEK-293T cells. It was expected that the VSV surface glycoprotein facilitates the entry of RINNL4-3 virions into the host cells, therefore any replication capability would be detectable. The cells were infected with SCR and RINNL4-3 virions pseudotyped by VSVG. Results of a quantitative p24 assay demonstrated no increase in the p24 load of the supernatant of RINNL4-3-infected HEK-293T cells after 4 days, whereas a 25-fold increase of the p24 load was observed in the SCR-infected cells (fig. 3).

HIV glycoprotein biological activity

Syncytium formation is an indicator for the biological activity of the HIV Env. To study the biological activity of RINNL4-3 Env, MT-2 cells were transduced by VSVG pseudotyped SCR HIV containing the RINNL4-3 provirus. MT-2 cells are known to be very

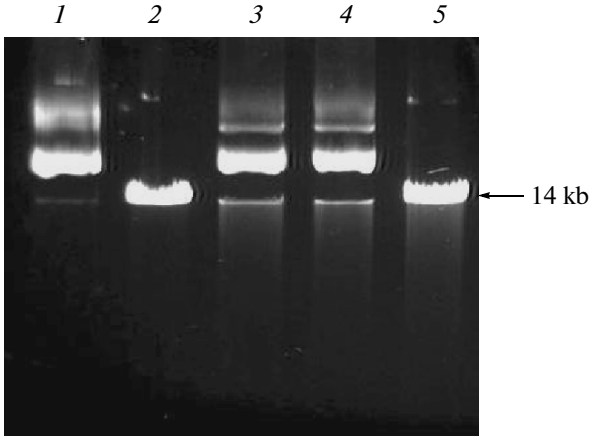

Fig. 2. Confirmation of the authenticity of the plasmids. Lane *1* – the pNL4-3; *2* – the BamH I digested pNL4-3; *3* – undigested pRINNL4-3; *4* – the Age I digested pRINNL4-3; *5* – the BamH I digested pRINNL4-3. As it can be seen in lane *4*, the pRINNL4-3 is not cut by Age I. Also as it can be seen in lane *5*, pRINNL4-3 does have its normal size.

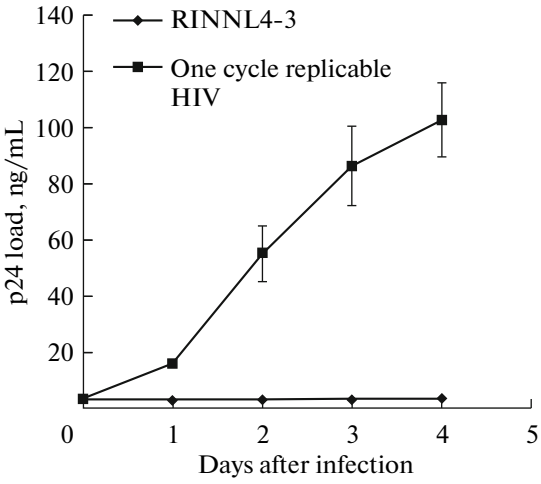

Fig. 3. Assay of replication activity of the VSVG pseudotyped single-cycle replicable (SCR) and RINNL4-3 virions in HEK-293T cells. After 4 days post infection there was no increase in the p24 load of the RINNL4-3 viron infected cells supernatant, however there was a detectable rise of the p24 load in the SCR VSVG pseudotyped virus infected cells starting 24 h post infection.

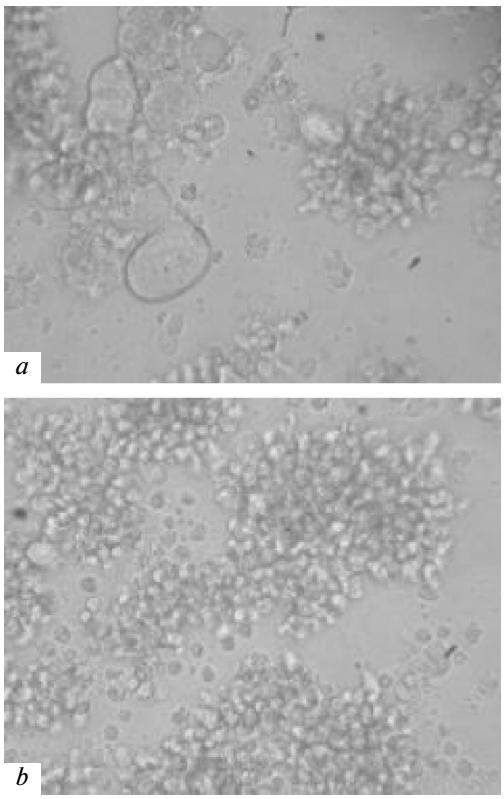

Fig. 4. MT-2 cells transduced with the HIV RINNL4-3 provirus (4 days post infection). *a* – Cells transduced with SCR HIV virions containing the RINNL4-3 provirus. Syncytiums were formed due to production of active envelope glycoprotein (Env). *b* – The control cells which were not infected.

sensitive to the Env mediated fusion. Syscitium formation was 80 times higher in the RINNL4-3 provirus transduced MT-2 cells than the negative control (fig. 4). According to the data, one can suggest that the glycoprotein coded by RINNL4-3 is biologically active and has a native conformation.

Evaluation of specific antibody response to VLPs

Sera of the mice immunized with the RINNL4-3 VLP and control animals were obtained as described above. As it can be seen in figure 1, sera of the negative control groups did not show any specific reactivity to the HIV-1 antigens, while almost all of the mice immunized with VLPs of HIV demonstrated an IgG antibody response 6 weeks after the first injection. However, titres of antibodies rose rapidly after the second injection in all of the immunized mice. The time course of the anti-Env-antibody response in the immunized mice is shown in figure 1*a*. Mouse number 4 had the highest IgG immune response, so that the same ELISA test for a series of the mouse serum dilutions was conducted. Results indicated that dilution rates at around 1 : 800 were still detectable (above the cut-off value) (fig. 1*b*).

DISCUSSION

HIV enzymes, RT and IN, have been well characterized. In previous studies it was shown that mutations in their catalytic domain can abolish or highly reduce virus replication [14, 15]. Hence, these genes would be among the best candidates for designing an inactive HIV clone.

Cys-to-Ser substitution at position 130 of the HIV-1 NL4-3 integrase resulted in production of inactive virions [21]. Also, replication of HIV-1 carrying point mutations in the HHCC-motif of IN was blocked at a step after adsorption/entry and prior to the initiation of reverse transcription [14]. Diallo et al. [22] and Wei et al. [23] provided biological and biochemical evidence that the Met184Val substitution in RT blocks the function of this enzyme. All these results show that virions containing inactivated RT and IN enzymes are defective in replication.

We have designed and prepared a mutant HIV-1 provirus and named it RINNL4-3. Four nucleotides to the middle of the *pol* sequence of the provirus, which shifted the ORF of RT and IN sequences, were added. Virions produced from this provirus were expected to lack RT and IN enzymes but to contain all other HIV proteins. The syncytium formation assay demonstrated that RINNL4-3 possess biologically active Env. The RINNL4-3 virions displayed ability of the wild type HIV for production of virion proteins, assembly of virion particles, maturation of virions, recognition of target cells and fusion of its envelope to the target cell membrane, but were not able to reverse transcribe and integrate the provirus into the target ge-

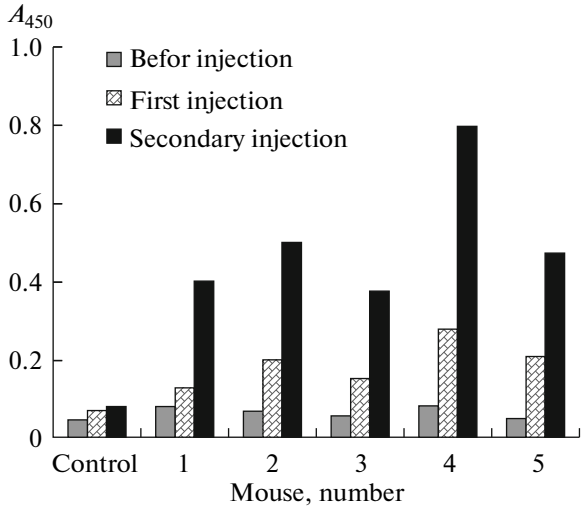

Fig. 5. Anti-Env HIV-1 humoral immune response in BALB/c mice induced after immunization with HIV-VLPs. Control mouse was not immunized.

nome. RINNL4-3 can be used for research at the lower biological containment levels without a danger of contamination or infection. Also, such an inactive virus could be a chance for vaccine investigations.

It was earlier shown that VLP-based vaccines induce a high-titre antibody response and can elicit both systemic and mucosal immune responses [24, 25]. Vaccination of monkeys with Gag-containing VLPs caused strong stimulation of the cellular immune response [26]. HIV Env protein embedded in the viral envelope of inactive HIV particles were more immunogenic and could trigger a higher antibody response as compared to soluble forms of Env as antigens [5]. At intranasal vaccinations of the BALB/c mice (on day 1, and weeks 3 and 6) with purified VLPs and soluble Env it was shown that mice vaccinated with VLPs elicited high cellular and humoral immunity to Env than those vaccinated with soluble form of the antigen [7]. In the latter study, a broadly neutralizing antibody response was seen only in mice which were stimulated with Env in the context of VLPs [7]. It was reason to suppose that the native structure of the Env remains unchanged only in composition of a lipid membrane that is like one in original HIV particles [4, 5].

The RINNL4-3 virion contains all of the HIV structural and surface proteins in addition to the viral lipids and nucleic acids. These inactive HIV virions were able to stimulate both the cellular and the humoral immune responses [10, 24, 27]. Data obtained in this study showed that the RINNL4-3 glycoprotein is able to efficiently induce syncytium formation among MT-2 cells (80 times higher than the control). The considerable fusion activity of RINNL4-3 Env implies the native conformation of this antigen in the envelope of RINNL4-3 virions. According to this result, the RINNL4-3 VLPs are expected to effectively trigger the neutralizing antibodies response against HIV. Our data showed that vaccination of BALB/c mice with RIINL4-3 resulted in a humoral response with a high antibody titre. All of the immunized mice produced high titres of antibodies against the HIV surface glycoprotein (fig. 5). It should be noted that HIV components deleted from the RINNL4-3 display a low immunogenicity for the HIV-1 specific CD4 response [28]. RINNL4-3 could be a promising immunogen VLP candidate for designing HIV vaccines.

Native structure of the mutated virions and their effective production in cell culture are the two major advantages of developed RINNL4-3, along with its prominent safety. Therefore, it could be used for a wide range of research projects on the HIV life cycle or even on the pathogenesis of HIV. We reported previously on a possible usage of pRINNL4-3 for the production of HIV SCR virions [20]. Improved biological safety level 2 (BSL2) laboratories would be sufficient to support research on the mutant virions because of their defect in replication.

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