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Production and Evaluation of Immunologic Characteristics of mzNL4-3, a Non-Infectious HIV-1 Clone with a Large Deletion in the Pol-Sequence[1]

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Abstract—Inactivation of integrase (IN) and reverse transcriptase (RT) can revoke the replication of HIV virions, and non-infectious HIV particles are desirable virus-like particle (VLP) vaccine candidates. Here, we produced inactive HIV-1 particles fit for vaccine and virological purposes by introducing a mutation into the *pol* sequence. Proviral DNA (pNL4-3) was cut at two points in the *pol* region using the BalI restriction enzyme and then religated. HEK 293T cells were transfected with the resultant plasmid (pmzNL4-3) to produce mutated virions. To confirm a production of VLPs and evaluate their biological activity the p24 load and syncytium formation (MT2 cells) were analyzed. The assay indicated that mzNL4-3 virions were assembled and contained functional envelope glycoproteins (ENV). In addition, mzNL4-3 virions were able to infect neither the MT2 nor HEK 293T cells. Furthermore, the immunogenicity of VLPs was investigated in a mouse model. According to the data on vaccinated mice, the titer of ENV-specific antibodies rose rapidly after a boosting injection. Moreover, lymphoid cells extracted from these mice proliferated after exposure to the antigen. The mzNL4-3 virus particles possessed immunogenic antigens of HIV and can effectively trigger humoral and CD4 immune responses. Non-infectious mzNL4-3 virions may also be used in biomedical experiments for improving the biological safety conditions. Moreover, the mzNL4-3 seems to be a promising candidate for further HIV-1 vaccine investigations.

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Keywords: human immunodeficiency virus, virus-like particles, reverse transcriptase, integrase, envelope glycoproteins, HIV-1 vaccine

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is a member of the retrovirus family and contains two copies of viral RNA[1]. The pre-integrating complex (PIC) is released into the target cell's cytoplasm after fusion of the virions envelope to the cell membrane. The PIC encompasses proviral RNA and integrase (IN) and reverse transcriptase (RT) enzymes as the major parts. The RT synthesizes dsDNA using the viral RNA as a template and then IN inserts this provirus DNA into a host chromosomal DNA [2]. Afterward viral proteins will be produced by cellular transcription and translation machineries. HIV infection causes the acquired immunodeficiency syndrome (AIDS) which is a chronic illness characterized by a decrease in the CD4+ lymphocyte count. The main targets of HIV virions are the CD4+ cells, and a chronic immune deficiency after depletion of CD4+ cells is seen in HIV infection. This process results in death due to tumors and opportunist infections [3].

Humoral and cellular immune responses to HIV infection play important roles during HIV pathogenesis [4, 5]. A strong cell mediated immune response (CD8 response) against HIV antigens starts during the first weeks of the infection [5]. The initial cellular immune response correlated with the viral load decline after the first viremia [6]. Production of antibodies which are able to neutralize the virus (neutralizing antibodies) is necessary for an effective humoral response [7]. HIV virions' surface glycoproteins (ENV) are the major target of neutralizing antibodies [8]. Recombinant forms of ENV are not able to trigger

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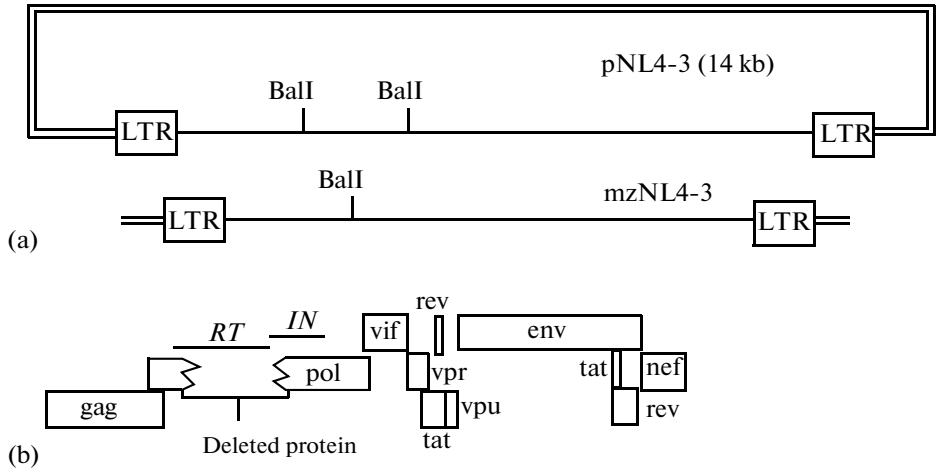

Fig. 1. Plasmid construction by introducing deletion mutation. (a) Restriction sites of the BalI (nucleotide 2620 and 4551) in the HIV-1 provirus. (b) Cutting NL4–3 provirus from these sites and then religating the blunt ends results in a deletion of 1931 nucleotides between BalI sites.

a broad production of neutralizing antibodies. Data from phase III clinical trials indicated that recombinant gp120 vaccination cannot influence disease progress or protect people [9]. The inability of recombinant ENV antigens to induce a broad production of neutralizing antibodies is due to the differences between the structure of ENV complexes in the virus particles and the particle-free recombinant ENV units [8]. Previous data showed that only ENV complexes in the composition of a virus-like particle (VLPs) or the envelope lipids can trigger broad production of ENV neutralizing antibodies [10, 11].

Inactive HIV virus particles that contain immunogenic structural and surface antigens in their native forms are suitable for vaccine investigations [8, 12]. As it was shown in previous studies, inactive and attenuated HIV virions are able to trigger protective cellular and humoral immune responses [13, 14].

Former studies have shown that inactivation of IN and RT enzymes can abolish the HIV virions replication. Even a point mutation in the catalytic domains of these enzymes (single amino acid substitution) can abolish their function [15, 16]. A non-covalent interaction between HIV Gag-polyprotein and the full-length viral RNA genome is necessary for the RNA packaging and, therefore, assembly of the virus particles [17, 18].

In the current study, we report on production, biological and immunological activity of a novel mutant HIV-1 clone (mzNL4-3), which has been designed by deletion of *RT* and *IN* sequences.

EXPERIMENTAL

Plasmid and deletion mutant construction. pNL4-3 is a recombinant provirus of HIV-1 LAV and NY5 strains cloned in a pUC18 vector and was used as a provirus source [19]. pLOX-CWGFP, pMD2.G and psPAX2 plasmids (purchased from Addgene) were used in this study to produce pseudotyped virions with VSV-G (vesicular stomatitis virus surface glycoprotein) in their envelope.

The BalI (Mls1) restriction endonuclease (Fermentas) was used to perform the deletion mutation [20] (Fig. 1). The BalI recognizes and cleaves TGGCCA sequences. This restriction enzyme cut the pNL4-3 plasmid in two sites at positions 2620 and 4551, after that the blunt ends of the digested pNL4-3 plasmid were ligated using the T4 DNA ligase (Fermentas).

Clones of mzNL4-3 were investigated to confirm the deletion mutation and its size. The pmzNL4-3 was digested with BalI, and products obtained were analyzed by electrophoresis on a 0.7% agarose gel. The size of the mutated plasmid (pmzNL4-3) was assessed after linearization.

Cell culture and production of viruses. The cells were maintained as previously described in DMEM or RPMI 1640 (Chemicon) supplemented with 15% fetal bovine serum (FBS, Gibco), penicillin G (100 U/mL), streptomycin (100 mg/mL), glutamine (2 mM) and HEPES (25 mM) [21].

HEK 293T cells were transfected using the polyfect reagent (Qiagen). The cells were placed in 24-well plates (7×10^4 cells/well) on the day before the transfection. The medium was replaced with 300 μL of fresh medium 3 h before the transfection. Subsequently, the transfection complex was prepared by mixing 400 ng of DNA and 4 μL of polyfec according to the manufacturer's protocol. At 36, 48 and 72 h post transfection, the virus-containing supernatant was harvested, pooled and stored at 4°C [22]. The supernatant was clarified using centrifugation at 10000 × g for 5 min. Virions were harvested by centrifuging at 60000 × g for 90 min at 5°C and then resuspended by gentle vortexing in 2 mL of DMEM overnight. Virus

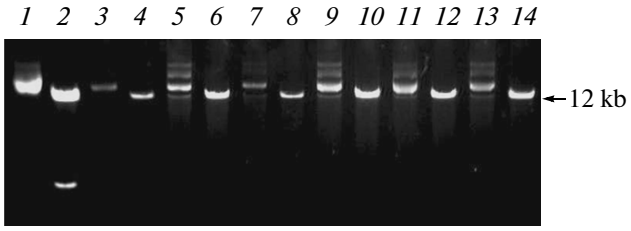

Fig. 2. The authenticity of the mutation as confirmed by BalI digesting the purified plasmids isolated from 6 clones. Lane *1*, pNL4-3; lane *2*, BalI digested pNL4-3; lanes *3–14*, undigested and BalI-digested pmzNL4-3 from 6 different clones.

stocks were measured for p24 content (HIV p24 ELISA, Biomeriux, France) and then stored at −70°C. To prepare VSV-G pseudotyped mzNL4-3 virions, 293T cells were co-transfected with pmzNL4-3 and pMD2.G.

Production of single cycle replicable and control virions. Single cycle replicable virions were produced by co-transfection of HEK 293T cells with pmzNL4-3, psPAX2 and pMD2G plasmids [22, 23]. Note, psPAX2 plasmid codes HIV polyproteins (Gag-Pol and Gag), but not virus RNA and surface proteins. These virions are able to replicate for no more than one cycle, and, therefore, are considered as the replication control [22].

Supernatants of 293T cells co-transfected with pLOX-CWGFP and psPAX2 were used as a virion production control. These virions contained no surface proteins in as much as pLOX-CWGFP, and psPAX2 only code the packaging RNA and internal proteins for newly budding virons. These virions were used in this study to compare pmzNL4-3 with other plasmids in the term of viral production capacity.

Measuring the replication ability and syncytium formation. HEK 293T and MT-2 cell lines were used as the infection targets. The cells were seeded at the density of 6×10^4 cells per well in the 24-well plates containing 350 μL of fresh medium and infected with 700 ng of p24 HIV virions for 16 h. The cells were washed three times with PBS and placed in 500 μL of fresh medium, and the 293T cell supernatants were collected every 24 h post infection, whereas the MT-2 cell supernatants were harvested every three days for 21 days. The p24 load in the cellular supernatants was evaluated by quantitative p24 ELISA (HIV p24 ELISA, Biomeriux).

Syncytium formation was measured by counting the number of syncytiums formed in five fields using a light microscopy. The cells were infected with 4 μg of p24 HIV virions, and the synsytium was counted 72 h post infection [24].

Mice immunization and evaluation of antibody response. Female BALB/c mice aged 6–8 weeks were injected with 50 μg of purified mzNL4-3 VLP in complete Freund's adjuvant. Injections were done subcutaneously (s.c.). After three weeks, the same dose of antigen with incomplete Freund's adjuvant was s.c. injected as a booster. Blood samples were taken from the retroorbital sinus at weeks 0, 5, 8 and stored prior to analysis at −20°C. The antibody response to the HIV ENV was evaluated using quantitative ELISA (Biomerieux).

Lymphocytes proliferation assay. Spleen cells were extracted from the immunized mice under sterile conditions 8 weeks after the first injection. The cells were placed in PBS containing 4% FBS. Red blood cells were lyzed and cell suspension was adjusted to 5×10^6 cells/mL. Afterward the cell suspension (100 μL) was cultured in each well of the 96-well plate with 4 μg of the virus for 72 h. Phytohemagglutinin-A (PHA 6 μg/mL; Gibco) was used as the positive control. Cell proliferation was measured by addition of 30 μL of methabenzthiauron (MTT) into each well. The plates were incubated at 37°C for 5 h, and then centrifuged at 3000 × g. The supernatant was removed carefully, and Formazan crystals were solubilized by adding 100 μL of dimethyl sulfoxide into each well. After 15 minutes of incubation, the absorbance of each well was read at 570 nm using an ELISA plate reader Biotek EL340 (USA).

RESULTS

Production of mzNL4-3 Virus Particles

In order to select the best pmzNL4-3 clone, DNA was extracted and digested with BalI. The deletion mutation in pmzNL4-3 clones was determined on the base of agarose gel fractionation results (Fig. 2). Then an ability of the selected pmzNL4-3 clones to produce virus particles was evaluated. The cells were transiently transfected with six pmzNL4-3 clones. Forty eight hours later, the supernatants were harvested and analyzed for the p24 load using quantitative p24 ELISA. The results showed that pmzNL4-3 from clone number 2 had a higher potential for production of mzNL4-3 virions (Fig. 3). A virus particle production in psPAX2 and pLOX-CWgfp transfected cells (GFP reporter virions) was considered as controls.

Infectivity of mzNL4-3

Infectivity of mutant virions, mzNL4-3, was evaluated in two cell lines (HEK 293T and MT-2). MT-2 cells are competent to be infected by HIV virions via HIV envelope glycoproteins (ENV). Rreplicable HIV virions can be grown in this cell line. HIV single cycle replicable virions which were produced from pmzNL4-3, psPAX2 and pMD2G transfected HEK 293T cells were used as a positive replicable control [22]. No rise in the p24 load was observed in the supernatants of mzNL4-3 infected MT-2 cells even after 21 days, whereas the infection of MT-2 cells with single cycle replicable HIV virions resulted in an increase of the p24 load in the supernatant in a few days.

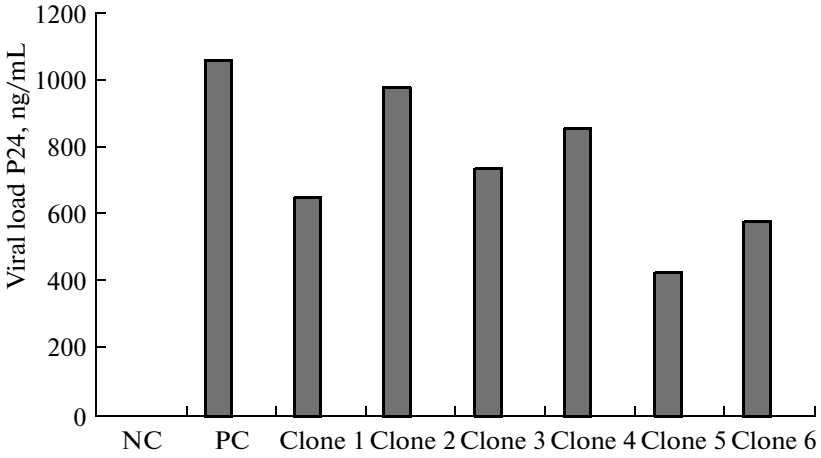

Fig. 3. Capability of the mzNL4-3 clones for virus production. VLP-production was evaluated as the p24 content in a supernatant of transfected cells (clones 1–6). The GFP-reporter HIV producer and the non-transfected cells were considered as a positive (PC) and negative (NC) control, respectively.

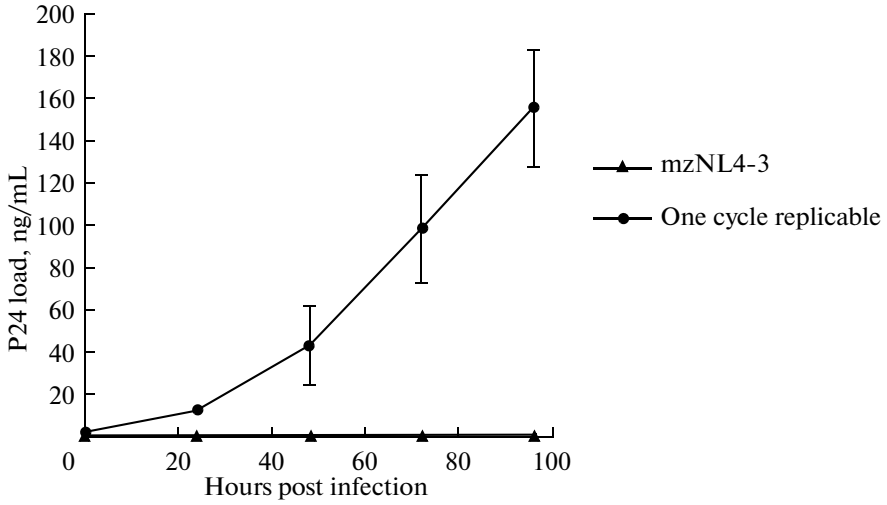

Fig. 4. Comparison of the replication rate of VSV-G pseudotyped one cycle replicable HIV and mzNL4-3 virions in HEK 293T cells. The p24 content in the cell supernatant was monitored daily until 4 days post infection.

Infectivity of the VSV-G pseudotyped mutant virions, which displayed high infection ability because of presence of the surface glycoprotein of VSV, was measured in HEK 293T cells. The cells were infected with VSV-G mzNL4-3 or single cycle replicable HIV virions. The p24-quantitative ELISA did not reveal an increase of the p24 content in the mzNL4-3 infected cell supernatant (until 4 days), whereas a roughly eightyfold increase was found in the case of single cycle replicable HIV infected HEK 293T cells over the same time interval (Fig. 4).

Biological Activity of mzNL4-3

MT-2 cells are susceptible to cell-cell fusion in a way that they form a syncytium after being infected by HIV [24]. Syncytium formation is a marker of biological activity of the ENV glycoprotein. Syncytium formation was measured in MT-2 cells transduced by VSV-G pseudotyped HIV virions containing the mzNL4-3 provirus and non-transduced MT-2 cells as a control. As it is seen from the results presented in the Fig. 5, a syncytium formation in mzNL4-3 transduced MT-2 cells was 86 fold higher compared with that in the control (Fig. 5).

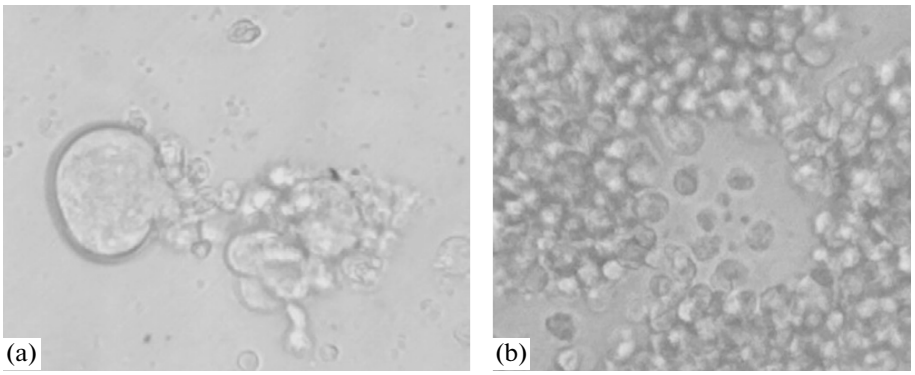

Fig. 5. Syncytium formation in mzNL4-3 transduced MT-2 cells 3 days post infection. (a) Cells were transduced with single cycle replicable HIV-1 virions containing the mzNL4-3 provirus. (b) The non-infected (negative control) cells. Magnification scale is 400×.

Immune Response to the mzNL4-3 in Mice

Sera and spleen cells of mzNL4-3 VLP immunized and control BALB/c mice were obtained as described above. No specific antibody reaction to HIV-1 ENV was detected in sera of the control, adjuvant-injected, group (mouse 1), while almost all of the mice immunized with mzNL4-3 VLPs demonstrated a high IgG antibody response after 8 weeks from the first injection (Fig. 6). Furthermore, antibody titer rose rapidly after the second injection in the immunized group. The time course of the anti-ENV response for this mice group is shown in Fig. 6a. Mouse number 4 had the highest IgG immune response, so that the same ELISA test for a series of serum dilutions of this mouse was conducted. Results indicated that dilution rates at around 1 : 800 were still detectable (above the cut-off value) (Fig. 6b).

A lymphocyte proliferation assay was performed using the MTT assay. The lymphocyte proliferation of all mice immunized with adjuvant–VLP was higher than that in control groups ($P < 0.002$; Fig. 6c).

DISCUSSION

HIV enzymes, IN and RT, were well-characterized. As it was shown earlier [15, 16], introducing mutation(s) into a catalytic domain of one of the enzymes can highly reduce or abolish virus replication. Hence, *RT* and *IN* genes would be good targets for designing a non-infectious HIV clones.

Results of previous studies showed that a substitution at position 130 (Cys-to-Ser) in the IN sequence of the NL4-3 provirus resulted in production of inactive HIV VLPs [25]. Also, HIV-1 virions carrying a point mutation in the HHCC-motif of the IN are non-replicable. These IN-negative virions are unable to complete their replication cycle at the step succeeding entry and reverse transcription [15]. In 2003, Diallo et al. [26] and Wei et al. [27] showed that the M184V substitution in RT blocked the function of the enzyme. The IN and RT enzymes have critical roles during the HIV virion's life cycle, so that virions containing inactive IN or RT are replication defective.

Here, a mutant HIV-1 clone was produced and named mzNL4-3. A large segment (1931 nucleotides) was removed from the middle of the *pol*-gene of the NL4-3 provirus which deleted 96% and 34% of the RT and IN sequences, respectively. Virions produced by this provirus were expected to contain all HIV antigens except the *RT* and *IN* enzymes. HIV mzNL4-3 was designed to have the capacity for virion protein production, assembly of viral particles and their maturation, recognition and fusion to the target cells' membrane, while it would lack the ability of reverse transcription of the virus genome and its integration into the host one. Results obtained indicated that mzNL4-3 virions may be produced effectively and are completely non-infectious. Moreover, results on the syncytium formation assay showed that mzNL4-3 contains biologically active ENV complexes. The mzNL4-3 particles could be a promising chance for lowering the biological safety levels without contamination and infection danger for HIV-based researches. Additionally, such non-infectious virus particles would be a candidate for vaccine development investigations.

As it was shown earlier, VLP-based vaccines trigger a high-titer antibody response and can elicit both mucosal and systemic immune responses [28, 29]. Monkeys immunized with a Gag-containing VLP showed strong activation of the cellular immune response [30]. ENV complexes embedded in the envelope of inactive HIV particles are more immunogenic and can induce a higher antibody response as compared to the soluble monomeric antigens [8]. Intranasal immunization of BALB/c mice with the purified HIV VLPs and soluble ENV (on day 1, weeks 3 and 6) showed that VLPs induce a higher level of cellular and humoral immune response than the ENV antigens do [10]. In the mentioned study, broad production of neutralizing antibodies was seen merely in mice immunized with ENV-VLPs [10]. Obviously, structure of HIV envelope glycoproteins in the composition

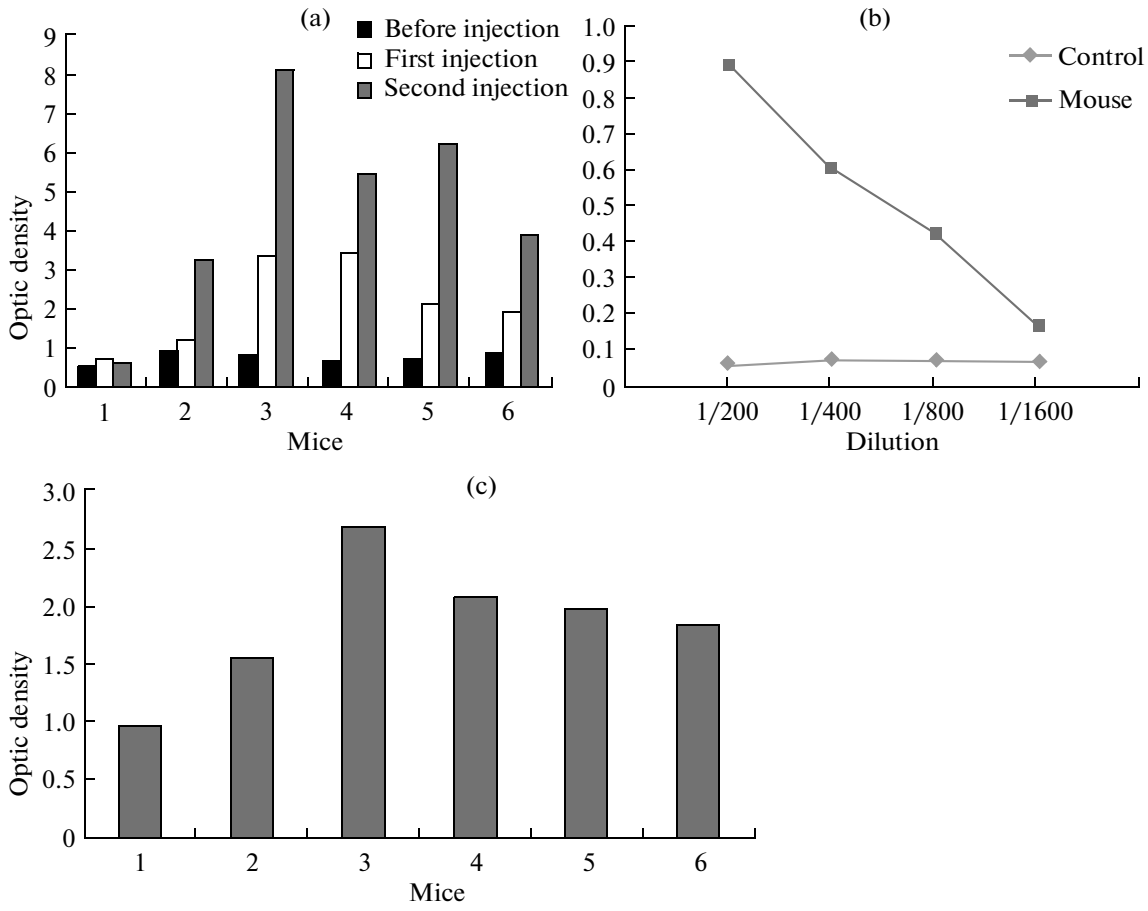

Fig. 6. Anti-ENV antibody and spleen cells proliferation response in mice immunized with mzNL4-3 and Freund's adjuvant (mice #2–#6) as compared with mouse #1, injected only by the adjuvant. (a) The presence of anti-ENV antibodies in sera samples collected from mice at weeks 0, 5 and 8 was investigated using ELISA. (b) The antibody titer of sera from the most responsive mouse #3 was measured in several dilutions. (c) The reactivity of immunized mouse spleen cells to mzNL4-3 virions was evaluated with the MTT method.

of the VLP lipid membrane reminds their structure in native HIV virion, and it seems to be a main reason for high immunogenicity of ENV-VLPs [8, 31].

Virus particles formed by the mzNL4-3 contain surface and structural antigens in addition to viral nucleic acids and lipids. As mentioned above, inactive HIV-1 virions would be able to stimulate both humoral and cellular immune responses [28, 29, 32]. Here, we have demonstrated that mzNL4-3 glycoproteins are able to efficiently trigger the formation of syncytiums in MT-2 host cells (86-fold better than the control). Thus, the mzNL4-3 ENVs are biologically active antigens and, accordingly, have a structure arrangement similar to that in native virus partivles. The presence of correctly curtailed (folded) ENVs on the surface of mzNL4-3 particles makes them effective antigens for triggering a broad production of HIV-neutralizing antibodies. The data show that BALB/c mice immunized with mzNL4-3 elicit a high humoral response with a significant antibody titer. All mzNL4-3 immunized mice had a high titer of antibodies specific for HIV ENV (Fig. 6). Spleen cells isolated from immunized mice displayed a high reactivity for mzNL4-3 VLPs that supports efficiency of the last for inducing the CD4 immune response. It should be noted that deleted components of the virion, IN and RT, are the less immunogenic ones for the HIV-1 specific CD4 response [33]. Thus, the mzNL4-3 is a promising immunogenic HIV VLP and can be considered as a candidate for designing HIV vaccines. Performing a more detailed investigation in this respect is the next phase of our research.

The mzNL4-3 VLPs contain immunogenic HIV-antigens and can be effectively produced in cell culture. These are two major advantages of the mzNL4-3 besides its prominent safety. HIV mzNL4-3 could be used by researchers in a wide range of studies on the virus life cycle or even pathogenesis. As mzNL4-3 VLPs are non-infectious, the improved biological safety level 2 would be sufficient and effective for supporting corresponding researches. We consider the design and production of mzNL4-3 clone as the first

step for a further research on development of anti-HIV drugs and vaccines, at that in biological safety conditions available in Iran.

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The authors declare that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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