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Elicitation of neutralizing antibodies by intranasal administration of recombinant vesicular stomatitis virus expressing human immunodeficiency virus type 1 gp120

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Abstract

Recombinant viral vectors are useful tools for AIDS vaccine development. However, expression of HIV-1 envelope genes using viral vectors has not been successful in the induction of potent neutralizing antibodies in vivo. We took advantage of the strong immunogenicity of vesicular stomatitis virus (VSV)-based vector and expressed HIV-1 HXB2 gp120 gene in the recombinant VSV. Our results showed that HIV-1 gp120 protein expressed by the recombinant VSV retained the native conformation of the protein to some degree and was recognized by two well-characterized broad anti-HIV-1 neutralizing monoclonal antibodies b12, 2G12. We further showed that only one time intranasal immunization with the recombinant VSV led to production of anti-HIV-1 anti-sera in mice. In addition, we found that the antisera had the ability to neutralize not only HXB2 envelope-pseudotyped HIV-1 viruses but also HIV-1 pseudotyped viruses with JRFL envelopes. These results suggest that HIV-1 gp120 expressed by the recombinant VSV, in combination with the route of intranasal administration, is an effective strategy to evaluate the immunogenicity of HIV-1 envelope protein and its variants in mice.

Keywords: HIV-1; Recombinant VSVs; Immunogenicity; Neutralizing antibodies; Vaccine

It is widely accepted that cellular and humoral immunity are both important for anti-HIV-1 vaccine development (reviewed in reference [1–3]). Studies have shown that HIV-1 infection and vaccination of macaques are capable of stimulating strong cellular immune responses [4,5]. In contrast to the cellular immune response, neutralizing antibodies against HIV-1 are more difficult to induce [2,3]. It has been a great challenge to obtain broad and high titer anti-HIV-1 neutralizing antibodies in AIDS vaccine development [2,3].

The target for neutralizing antibodies against HIV-1 is the trimer envelope protein on the native virion [6,7]. The structure of the HIV-1 envelope glycoproteins, particularly those of primary isolates, has evolved to minimize the elicitation and efficacy of neutralizing antibodies [8–10]. In principle, it is possible to genetically re-engineer the immunoevasive envelope protein and to convert it into the immunostimulatory vaccine antigen. Several reports have indicated that specific modifications introduced in the HIV-1 envelope may increase the immunogenicity and stimulate animals producing broadly neutralizing antibodies [11–14]. The modifications include loop deletion [11,12], introduction of unique Pro-Met mutation into V3 tip of HIV-1 envelope [13], and the removal of selected N-linked glycosylation sites [14]. Development of an optimally immunogenic envelope vaccine will require further more extensive modifications on HIV-1 envelope proteins and high-throughput screening of potential immunogens. The large amount required for HIV-1 envelope protein used in immunization or the length of time required for maturation of HIV-1 neutralization antibodies in DNA vaccination has constrained the development of high-throughput

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screening for immunogenicity of different HIV-1 envelope mutations. Therefore, methods that can induce more rapid and potent neutralizing antibodies are needed to test number of potential immunogens.

Of the multiple vaccine approaches explored to date, the most robust protective immunity is generated by live attenuated virus vaccines [15–17]. Live recombinant viruses have been used to evaluate the effectiveness of HIV-1 envelope protein in inducing protective immunity against HIV-1 (reviewed in [18]). Vesicular stomatitis virus (VSV), a member of the Rhabdovirus family, is a non-segmented, negative-strand RNA virus. The development of a system to recover VSV from cDNA has allowed the manipulation of VSV genome and the expression of foreign genes in recombinant VSV. A number of genes from different viruses have been inserted into the recombinant VSV vector and recombinant VSVs have then been produced and used as immunogens to immunize animals and these have stimulated strong immune responses against a particular virus [19–21]. The recombinant VSV vector has also been used in HIV-1 vaccine development [22]. In particular, the recombinant VSVs expressing HIV-1 89.6 Env and Gag protein have been used to immunize macaques and have been shown to generate protective immunity against the SHIV 89.6P challenge [23].

In the current study, we developed a screening strategy using an antigen-expressing recombinant VSV and a single dose of intranasal immunization of mice. We also used pseudotyped HIV-1 reporter viruses to determine the titers of neutralizing antibodies. Our results showed that this method could serve as an effective and rapid alternative approach to examine the effects of HIV-1 gp120 mutations on production of anti-HIV-1 neutralizing antibodies.

Materials and methods

Plasmids. Viral RNA was extracted from collected VSV (Indiana serotype, a gift from Dr. Michael A. Whitt, University of Tennessee) culture supernatant by Qiagen QIAamp RNA mini kits (Qiagen, Valencia, CA). Five fragments of VSV genome were generated by RT-PCR and assembled into a full-length cDNA. The pBluescript SK (+) (Strategene, La Jolla, CA) was modified by inserting T7 terminator from pET-28a (Novagen, Madison, WI) between *Xba*I/*Sac*I sites and was named as SK-Ter. The full-length cDNA of VSV was inserted between the T7 promoter and ribozyme sequence of hepatitis delta virus in SK-Ter [24,25]. Two complementary oligonucleotides containing the transcript stop/start sequences and a *Xho*I site were cloned into *Nhe*I site between G and L genes [26]. The resulting plasmid was designated as pVSV. The plasmids named pSK-N, pSK-P, and pSK-L were constructed by cloning N, P, and L genes into pSK-Ter at *Xho*I/*Xba*I sites [27]. The HIV-1 HXB2 gp120 gene was modified so that the signal peptide of TPA from pJW4304 (a gift from Prof. James Mullins at University of Washington School of Medicine) was used to substitute the signal peptide of HIV-1 HXB2 gp120 and the transmembrane region and cytoplasmic tail of VSV-glycoprotein (VSV-G) were added to the C terminus of HIV-1 HXB2 gp120. The fusion gene encoding HIV-1 HXB2 gp120 aa 32–505 was then cloned into pVSV plasmid using *Xho*I and *Nhe*I sites. The resulting plasmid was designated as pVSV-HXB2. Meanwhile, the green fluorescent protein (GFP) gene from pEGFP-N1 (Clontech, Palo Alto, CA) and luciferase gene were also cloned into pVSV, respectively (designated as pVSV-eGFP and pVSV-luc).

Recovery of recombinant VSV. BHK-21 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Nearly confluent cells (90% confluence) were infected with vTF7-3 (a gift from Prof. Bernard Moss at NIAID, NIH), a recombinant vaccinia virus expressing T7 RNA polymerase. The multiplicity of infection (MOI) was 10. Sixty minutes after infection, the cells were transfected with 4 μg each of pVSV-N and pVSV-P and 2 μg pVSV-L, and 10 μg pVSV-HXB2, pVSV-eGFP, pVSV-luc or pVSVFL(+) using a Lipofectamine 2000 transfection kit according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). The cells were incubated at a 37 °C, 5% CO_2 incubator for 48 h, and the cells on culture dishes were frozen/thawed three times. The supernatants were collected by centrifugation at 2000*g* for 10 min and debris was removed by passing through a 0.2 μm filter. The supernatants were added to fresh BHK-21 cells at a ratio of 5 ml per 10 cm dish and incubated with the cells for 48 h. One milliliter of each supernatant was collected from these cells and added to another plate of fresh BHK-21 cells. Recovery of recombinant infectious VSVs was performed 48 h later and confirmed by development of cytopathic effect (CPE) in BHK-21 cells. The recombinant VSVs were propagated in BHK-21 cells and stored at −80 °C. The titers of the recombinant VSVs were determined to be 10^8–10^9 pfu/ml using a standard plaque assay. The recombinant VSV-luc was determined by luciferase activity in infected BHK-21 cells on 24-well plates. Recombinant VSV-eGFP was determined for GFP expression under a fluorescence microscope.

Western blot. Recombinant VSV-HXB2 gp120 and VSV-eGFP were used to infect BHK-21 cells at a MOI of 10. After 10–12 h, the cells exhibited visible CPE, but still attached to the plates. Cells were gently rinsed with PBS and then lysed in 100 μl of 1× SDS loading buffer. Proteins were electrophoretically separated on 10% SDS–polyacrylamide gel (SDS–PAGE) and then transferred to the nitrocellulose membrane (Amersham, Piscataway, NJ). The membrane was blocked in 5% milk in TTBS (20 mM Tris–HCl, pH 7.5, 0.1 M NaCl, and 0.5% Tween 20) at 37 °C for 1 h and with 1:500 sheep anti-HIV-1 gp160 antiserum (obtained from the National Institute of Health AIDS Research and Reference Reagents Program) at 4 °C overnight, and then probed with an alkaline phosphatase-conjugated secondary antibody. The proteins on the membrane were visualized using the BCIP/NBT solution (Promega, Madison, WI).

Flow Cytometry. Wild-type VSVs and recombinant VSV-HXB2 were used to infect BHK cells. When the cells began to show CPE, occurring 8–10 h post-infection, the cells were collected, washed with PBS, and stained with human anti-HIV-1 monoclonal antibodies b12, 2G12, 17b, and 48d (obtained from the National Institute of Health AIDS Research and Reference Reagents Program) on ice for 1 h and then with FITC-conjugated goat anti-human IgG (Santa Cruz, Santa Cruz, CA). An unrelated human IgG (a gift from Dr. Johnny He of Indiana University) was included as an immunostaining control. The cells were analyzed by a flow cytometer (DakoCytomation, Fortcollins, CO).

Mouse immunization. Female BALB/c mice were housed in the Animal Center of Peking University School of Medicine and used under a protocol approved by an Institutional Animal Use and Care Committee. All animals were kept in filter-isolette cages in a BL-2 animal facility of Peking University School of Medicine. Five- to six-week-old mice were intranasally immunized with 20 μl (10^6 pfu) VSV-HXB2 diluted in DMEM. VSV-eGFP was used as immunization control, DMEM alone was used as mock. There were three groups in total and each group had 5–7 mice.

Serum preparation. Blood samples were collected at 0, 4, 5, 6, 7, and 9 weeks following immunization and allowed to clot at 4 °C overnight. Clots were removed by centrifugation at 10,000*g* for 10 min. The sera were then transferred to sterilized Eppendorf tubes and heat-inactivated at 56 °C for 60 min. All sera were stored at 4 °C.

Detection of anti-HIV-1 antibodies in the sera. The production of antibodies against HIV-1 envelope was detected by the binding ability of the mice sera to 293T cells transiently expressing HIV-1 HXB2 envelope gene. Twenty microgram of pSV7d-HXB2 was transferred (obtained from the National Institute of Health AIDS Research and

Reference Reagents Program) into a 10 cm dish of 293T cells by calcium phosphate-mediated transfection. After 24 h, the cells were collected and washed twice with PBS. Expression of HIV-1 envelope was determined by staining with b12 and 2F5 antibodies using FACS analysis, the normal human IgG with the same concentration used as a control. Mice sera were analyzed by FACS, the pre-immune sera used as the negative control.

Anti-HIV-1 neutralization assay. The sera's neutralizing activity was determined by incubating the pseudotyped HIV-1 viruses with the sera and monitoring the inhibition of the infectivity of these pseudotyped viruses, as described [28]. Briefly, HIV-1 luciferase reporter viruses (pNL4-3.Luc.R^-E^-) pseudotyped with HIV-1 HXB2, JRFL envelope protein were incubated with the sera at dilutions of 1:30, 1:60, and 1:120 at 37 °C for 1 h and then used to infect U87-CD4-CCR5 (JRFL) and U87-CD4-CXCR4 (HXB2) cells on the microplate wells (96-well flat bottom). HIV-1 pseudotyped luciferase reporter viruses without anti-serum treatment were included as controls. HIV-1-infected cells were allowed to incubate for 2 more days and then lysed. The luciferase activity was assayed using a Luciferase Assay System (Promega, Madision, WI) and a Wallac Microbetta 1420 Counter (Perkin-Elmer, Boston, MA). The neutralizing activity of the mouse sera against SARS-CoV was used as a control for the serum specificity using a similar assay [29]. In addition, each immune serum sample was compared to the serum of given mice before immunization to test nonspecific neutralization activity. Each neutralizing experiment was performed in duplicate and repeated at least twice. The neutralizing ability of the sera from each group was calculated by subtracting the mean luminescence in cells infected with HIV-1 pseudotyped viruses with serum treatment from that in cells infected with HIV-1 pseudotyped viruses without serum treatment and expressed as a fraction of that in cells infected with HIV-1 pseudotyped viruses without sera treatment. As the negative control, the reactivity of the immunized sera with SARS-CoV pseudotyped virus expressed by the infected rate; this was calculated by the mean luminescence of cells infected by SARS-CoV pseudotyped virus treated with immunized sera divided by the mean luminescence of cells infected by SARS-CoV pseudotyped virus without sera treatment.

Results

Construction of full-length VSV plasmid and viral recovery

Five fragments of VSV genome were obtained by RT-PCR and assembled into a full-length cDNA. The cDNA was cloned into modified pBluescript SK (+) vector under control of T7 promoter and T7 terminator. To generate a correct 3′ end of VSV, the ribozyme sequence of hepatitis delta virus was introduced downstream of VSV trailer sequence. In order to facilitate the cloning of foreign genes, a linker containing the minimal transcript start and stop sequences and restriction endonuclease sites was inserted between G and L genes. The whole plasmid was named pVSV (Fig. 1A) and successfully recovered infectious VSV (rVSV) as detailed under Materials and methods. The virus purified from infected cell supernatants shown to express five proteins L, G, M, P, and N (Fig. 1B), and had the same pattern as the wild-type VSV. To test if foreign genes could be expressed in the vector, we constructed and recovered recombinant VSV containing eGFP, the eGFP expression confirmed under a fluorescence microscope (data not shown). To confirm that the foreign genes were able to be expressed in this vector, we constructed and recovered recombinant VSV containing luciferase gene,

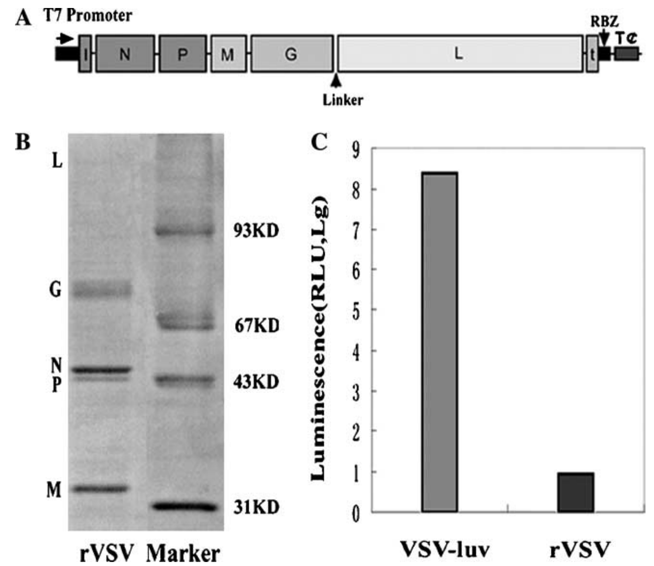

Fig. 1. Construction and recovery of recombinant VSV. (A) Schematic diagram of recombinant VSV vector. (B) Cell supernatants were collected 15 h after recombinant VSV infection. Viruses were purified by ultracentrifugation through 10% sucrose and then resuspended with PBS. Protein expressed by recombinant VSV was separated by 10% SDS–PAGE and visualized by staining with Coomassie brilliant blue. (C) Sixteen hours after infection by VSV-luc and VSV at MOI of 3, BHK-21 cells were lysed and measured for luciferase activity.

and then measured the luciferase activities in BHK-21 cells infected by VSV-luc, the luciferase activities in BHK-21 cells infected by VSV used as negative control (Fig. 1C). All the data indicated that pVSV could be used as a gene delivery carrier.

gp120 protein expression by the recombinant VSV viruses

We made a modified version of HIV-1 HXB2 gp120 by replacing its signal peptide with that of TPA and adding the transmembrane region and cytoplasmatic tail of VSVG to its C terminus. We first inserted modified HIV-1 HXB2 gp120 and eGFP gene in the VSV vector. We then successfully recovered recombinant VSV-HXB2, VSV-eGFP viruses, and wild-type VSVs after 2-3 passages in BHK-21 cells following the initial transfection. The BHK-21 cells inoculated with the recombinant viruses showed typical CPE within 18–24 h after 2–3 passages. The titers of recombinant VSV viruses were found to be in the same range as wild-type VSV viruses, i.e., between 10^8 and 10^9 pfu/ml, as assessed by the plaque assay. The gp120 expression in the recombinant VSVs infected cells was confirmed by Western blot assay using an anti-HIV-1 HXB2 gp160 antiserum (Fig. 2A). We also found that gp120 was expressed on the surface of the recombinant VSV-HXB2 infected cells, as the epitopes of gp120 on the cell surface were recognized by HIV-1 neutralizing antibodies b12, 2G12 by the cell surface staining and FACS analysis (Fig. 2B). This staining was specific, as no staining was detected on the cells infected with wild-type VSV.

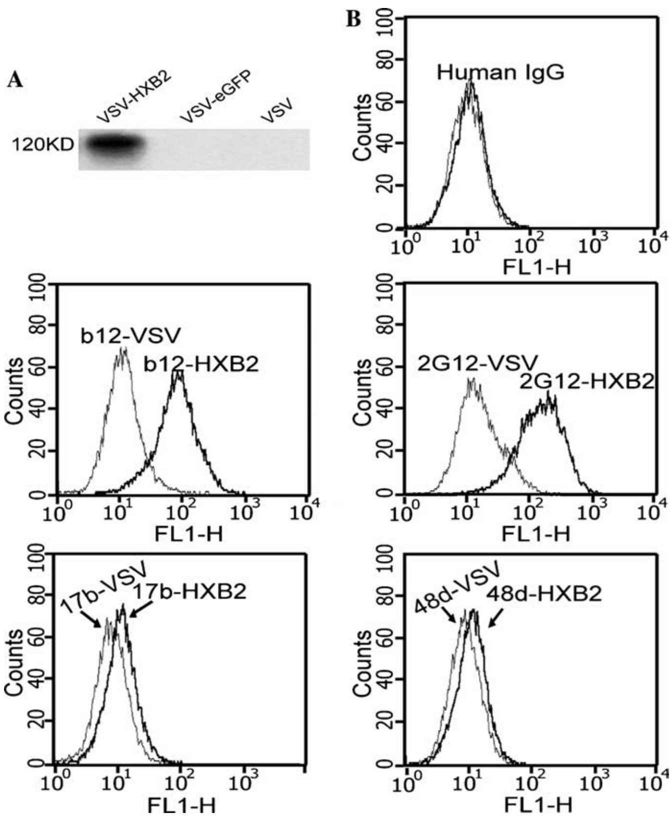

Fig. 2. Identification of gp120 expression in recombinant VSV infected BHK-21 cells. (A) Western-blotting analysis. (B) FACS analysis. The antibodies used are: normal human IgG (Unrelated antibodies), b12, 2G12, 17b, and 48d. On native gp120 protein the epitopes of b12 and 2G12 were well exposed, while the epitopes of 17b and 48d were cryptic.

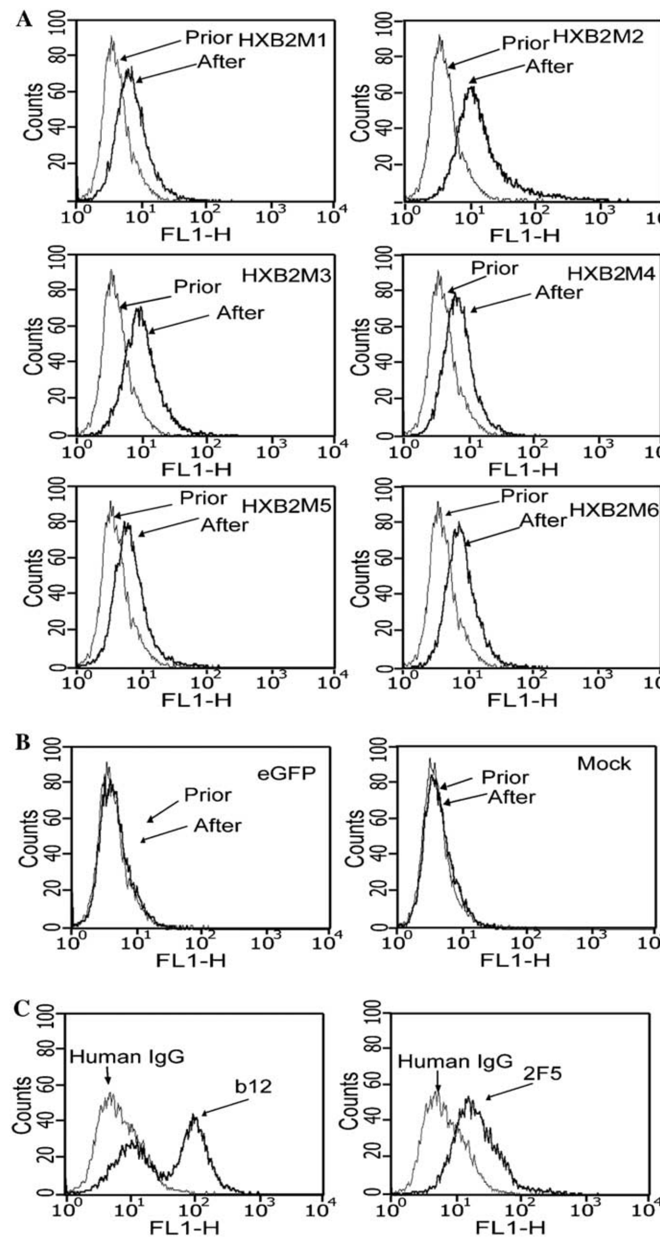

Fig. 3. Binding ability of the antisera with the cell surface-expressed HIV-1 HXB2 envelope protein. (A) Antisera of mice immunized with VSV-HXB2 (M1-M6) at 5 weeks. (B) Antisera of mice immunized with VSV-eGFP and DMEM (mock) as negative controls. (C) HIV-1 neutralizing antibodies b12 and 2F5 as positive controls.

Immunogenicity of recombinant VSV

One of the advantages of using recombinant VSVs was their ability to induce strong immune responses in vivo. Also, these viruses could be intranasally administered into mice in a single dose. Nevertheless, these recombinant viruses have also been shown to induce high titers of anti-VSV neutralizing antibodies due to high level of VSV-G protein expression within these viruses [30,31]. In agreement with these findings, our results showed that the sera obtained from immunized mice had high titers of anti-VSV neutralizing antibodies and inhibited infection of VSV-G pseudotyped viruses by over 90% at a dilution higher than 1:800 (data not shown).

To investigate the ability of recombinant VSV-HXB2 to induce anti-HIV-1 antibodies, we used sera from immunized mice from 5 weeks and preimmune sera as the primary antibodies to stain 293T cells transiently transfected by HIV-1 HXB2 envelope expressing vector. These cells were able to be stained by HIV-1 neutralizing antibodies b12 and 2F5 but not by normal human IgG. At a 1:50 dilution, the sera from mice immunized with VSV-HXB2 bound to 293T cells transfected by HIV-1 envelope gene. No sera from mice immunized with VSV-eGFP and preimmune sera showed the binding activity (Fig. 3). These data suggest that VSV-HXB2 immunization produced anti-HIV-1 antibodies.

Anti-HIV-1 neutralizing activity of the sera

To determine whether these obtained sera have any anti-HIV-1 neutralizing activity, we measured the inhibitory effects of these sera on HIV-1 infectivity. We performed a single-round HIV-1 infection of U87-CD4-CCR5 and U87-CD4-CXCR4 cells using HIV-1 luciferase reporter viruses treated with or without these sera. We also included anti-HIV-1 neutralizing antibodies b12 and 2F5 as positive

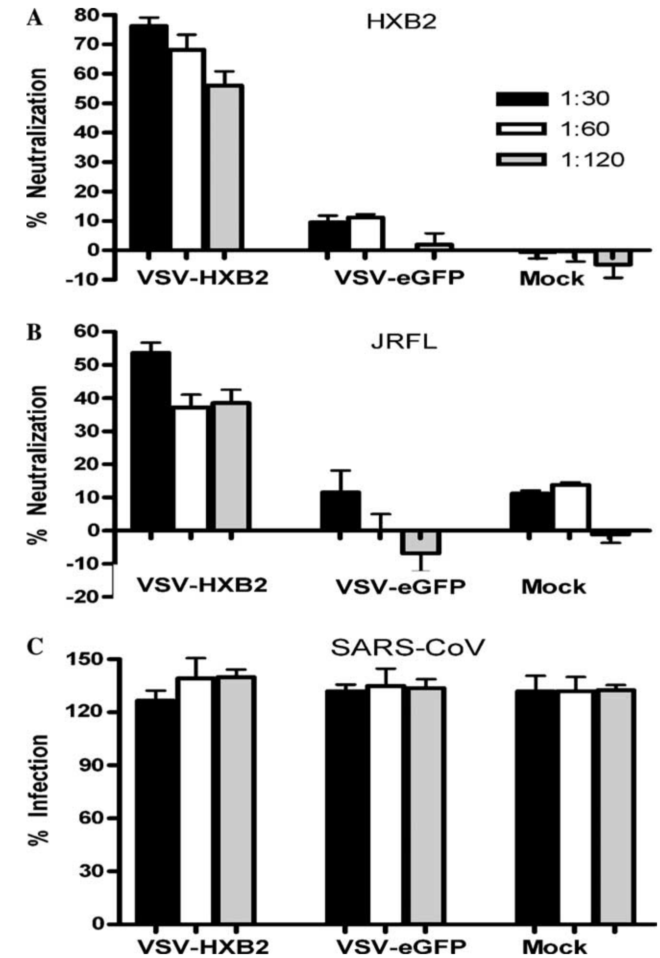

Fig. 4. Neutralizing assay of pseudotyped viruses. The mean and standard deviation in each group are shown at different dilution. (A) The neutralizing activity of immunized sera for HIV/HXB2 pseudovirus. (B) The neutralizing activity of immunized sera for HIV/JRFL pseudovirus. (C) The effects of the immunized sera on HIV/SARS infectivity as negative controls.

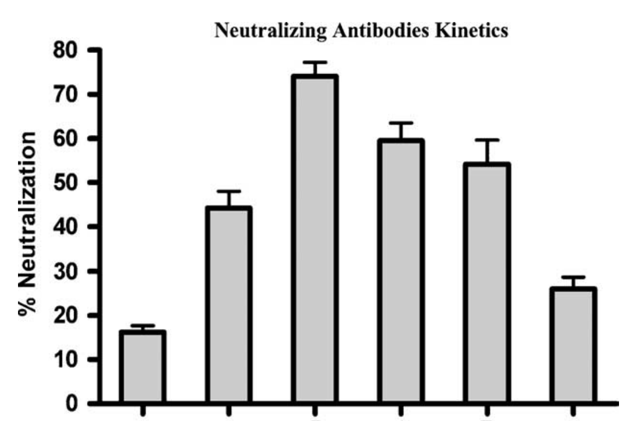

Fig. 5. The kinetics of neutralizing antibody response. The neutralizing activity of immunized sera (1:30 dilution) for HIV/HXB2 pseudotyped virus at 0, 4, 5, 6, 7, and 9 weeks after the immunization.

controls in these experiments. In agreement with published data [32], b12 neutralized HIV-1 HXB2, JRFL with an IC_{50} at 0.015, 0.041 μg/ml, and 2F5 neutralized with an IC_{50} at 0.018, 0.29 μg/ml, respectively. The sera from VSV-HXB2 gp120 immunization had anti-HIV-1 HXB2, and JRFL neutralizing antibodies (Fig. 4), with anti-HIV-1 HXB2 reaching its peak at week 5 after immunization (Fig. 5).

Discussion

We describe here a screening strategy based on recombinant VSVs to evaluate the immunogenicity of HIV-1 envelope. In this study, we confirmed that foreign genes can be engineered into VSVs, and the recombinant VSV expressing HIV-1 envelope can induce neutralizing antibodies by a single intranasal inoculation. The immunization of mice with recombinant VSVs expressing various HIV-1 envelope or HIV-1 envelope mutations and detection of neutralizing ability to HIV-1 isolates in a standard assay panel [33] serve as an effective and rapid alternative approaches to determine better immunogens for eliciting neutralizing antibodies. This strategy could facilitate HIV-1 vaccine development.

One of the advantages of the rVSV vector is the high titer of recombinant VSVs [27]. In our experiments, the recombinant VSVs expressing HIV-1 gp120, luc, and eGFP recovered by passage on BHK-21 cells 3 times have been determined by plaque assay. The titers were 1.2×10^8, 1.5×10^8, and 2.7×10^8 pfu/ml, respectively, the titer being lower than those of recovered wild-type VSVs which were 4.5×10^8 pfu/ml, but they are within the same range. Another advantage of VSV-based vector is that recombinant VSVs can stimulate strong cellular and humoral immune responses [19,20]. Recombinant VSVs expressing HIV-1 gag and envelope proteins can form virus like particles and induce high level CTL responses against HIV-1 gag and envelope [34,35]. In our experiments, the intranasal inoculation with recombinant VSV expressing HIV-1 HXB2 gp120 only once can induce antibodies binding HIV-1 envelope protein expressed on the 293T cell surface. The anti-sera of mice could neutralize HIV-1 HXB2 and JRFL pseudotyped HIV-1 reporter viruses. The titer neutralizing HXB2 measured as IC_{50} is about 1:120 and the titer neutralizing JRFL is about 1:30. All the sera specifically inhibit HIV-1 envelope pseudotyped viruses since they cannot inhibit SARS-CoV spike pseudotyped virus entry host cells. This neutralizing titer is at the same range as DNA immunization results of modified HIV-1 gp120 or gp140 by adding three copies of C_3d as molecular adjust. The DNA immunization needs boost DNA twice after the first immunization or boosted with protein after two times DNA immunization, so it requires at least 4 months to develop neutralizing antibodies [36,37]. After recombinant VSV immunization, there were detectable neutralizing antibodies at 4 weeks and peaked at 5 weeks. This short time of maturation of HIV-1 neutralizing antibodies makes this method very convenient for evaluating HIV-1 envelope protein's immunogenicity.

The recombinant VSVs express gp120 on the cell's surface of infected BHK-21 cells. As shown in Fig. 2, the gp120 expression can be identified by Western blot on the cell lyses and by FACS analysis with HIV-1 mAb b12, 2G12. These data confirmed that the modified gp120 on the cell surface kept the naïve conformations which may be important for inducing HIV-1 neutralizing antibodies. Although we detected the expression of gp120 in the infected cells, but there were no detectable gp120 on the recombinant virions. We pelleted the viruses and compared the viral protein expression within the recombinant viruses. We found that all recombinant VSV virions expressed the L, N/P, and M protein at similar levels to those of wild VSVs on a Coomassie-stained SDS–PAGE (data not show). However, we were not able to detect the presence of HIV-1 gp120 within the recombinant VSV virions. This result is consistent with previous data [34] that rVSVs expressing HIV-1 89.6 gp160 envelope at high level (30% of that of VSV G protein), but much lower gp160 (about 3% of VSV G protein level) was incorporated into recombinant VSV virions. The lower level of inoculation of HIV-1 envelope protein is quite different when compared with other foreign proteins expressed by rVSVs. As described previously, CD4 protein, measles virus hemagglutinin are both effectively incorporated into virions [27]. The lower incorporation may be due to the intracellular retention and degradation of HIV-1 envelope before it reaches the cell surface [34,38]. To further enhance the immunogenicity of HIV-1 envelope protein expressed by rVSVs, new recombinant VSV vectors encoding costimulatory molecules and cytokines or by expressing HIV-1 envelope protein covalently linked to C_3d will be developed and tested. With these improvements on VSV vector, the HIV-1 envelope antigen will be more effectively presented and easy to immunize. In addition, the neutralizing antibodies can be detected within a shorter time. The recombinant VSVs expressing HIV-1 envelope and envelope mutations will make a high-throughput comparison possible.

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