Research Article:
Expression of HIV-1 gag and env genes

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Abstract

Traditional vaccine methods have long been employed to control widespread infectious diseases, but so far, all commercially available vaccine strategies have been inadequate in efforts to develop an effective therapeutic HIV vaccine. However, recent advancements in immunological research have led to the generation of novel vaccine strategies, one of which is the recombinant virus vaccine, a method of particular interest that has shown promise in the clearance of HIV infection within HIV-positive patients who have retained immunocompetence. This study examined the stability of expression of HIV-1 genes, gag and env, through a recombinant virus vector, a recombinant vesicular stomatitis virus (VSV), a temperature-sensitive mutant genetically modified to contain the select HIV-1 genes (VSVInd(GML)HIV-1gag-env). For SDS-PAGE, cells infected with VSVInd(GML) temperature-sensitive mutants were incubated at 31 °C and 37 °C, representing permissible and semi-permissible growth conditions respectively. Western blot analyses were used to quantitate levels of protein expression of full VSV proteins, Gag, and Env using a primary rabbit antibody of anti-VSV anti-serum and a secondary anti-IgG from rabbit, a primary antibody of anti-p24 anti-serum and a secondary anti-IgG from rabbit, and a primary goat antibody, anti-gp120, and a secondary anti-IgG from goat, respectively. Results indicated that the VSVInd(GML) vector system allowed for high levels of expression of HIV-1 gag and env genes. It is known that the expression of these genes induce the production of major neutralizing antibodies and the stimulation of cytotoxic T lymphocytes, therefore this finding reveals the potential to use a genetically modified recombinant VSV as a universal vector for the development of recombinant virus vaccines. Specifically, the VSVInd(GML) mutant vector is thus an attractive candidate for the viral vector of a therapeutic HIV vaccine system.

Introduction

According to the World Health Organization report in 2013, HIV has infected over 70 million people and 36 million people have died from AIDS-related illnesses since 1981. Additional reports from United Nations AIDS disclosed that 2.3 million people were newly infected in 2012, adding to a total of 35.3 million people diagnosed with HIV infection as of 2013. HIV/AIDS is a rising world epidemic and our search for a method to control the spread of this disease has presented one of the most challenging problems of the past three decades.

Vaccines are one of the most effective methods of controlling infectious disease and thus a central focus for HIV treatment has been on efforts to develop an effective therapeutic vaccine that will clear HIV infection. There are three types of commercially available vaccine strategies used to prevent many different viral diseases. These include the live-attenuated virus vaccine, such as the measles and mumps vaccines, the killed whole-virus vaccine, such as the influenza and hepatitis A vaccines, and subunit vaccines, such as human hepatitis B virus and human papilloma virus vaccines. However, these three conventional vaccine strategies have so far proved ineffective for use as therapeutic HIV vaccine systems, effectively demonstrating our limited and largely empirically-based knowledge of these technologies and our lack of a comprehensive understanding of the immunological principles underlying widespread infectious diseases such as HIV. Recent advancements however, have generated novel vaccine strategies, one of which is the recombinant virus vaccine, a method that has shown to be promising in clearance of HIV infection within HIV-positive patients who have retained immunocompetence.

Recombinant virus vaccines depend on the use of a viral vector to carry genes of interest, such as HIV genes, and express them in the body to activate CD8 positive cytotoxic T lymphocytes (CTLs). Development of a therapeutic HIV vaccine to clear persistently infected...
cells depends on the induction of antibody-dependent cellular cytotoxicity and the activation of viral-specific CTLs—to destroy and clear HIV-infected cells. In particular, CTL response is considered a major immune defense mechanism required for recovery from nearly all viral infections. It is not desirable to use live HIV for CTL activation, but through a recombinant virus vaccine, it is possible to selectively express HIV genes that will activate HIV-specific CTLs.

The stimulation of HIV-specific CTLs also depends on the HIV genes expressed through the recombinant viral vector. HIV is a retrovirus with a genome expressing three major structural proteins, Gag (capsid protein, matrix protein), Pol (RNAse H, integrase, and protease) and Env (gp120 and gp41). HIV-1 gag and env genes in particular can play critical roles in the induction of adaptive-host immune responses. Gag, group-specific antigen, facilitates viral assembly and maturation. When gag is expressed without pol in recombinant virus-infected cells, the precursor Gag55 assembles virus-like particles (VLPs) that are non-infectious and mimic HIV virions. Env, envelope, codes for the Gp120 and Gp41 glycoproteins present on the surface of HIV as trimmers. Env precursor protein, Gp160, is synthesized in the endoplasmic reticulum and transported to the Golgi where it is cleaved into Gp120 and Gp41 by cellular proteases. The surface protein, Gp120, attaches to CD4 and chemokine receptors of susceptible cells to initiate infection. It was found that HIV-1 Gag particles carrying multiple T-cell epitopes of Gp120 induced strong CTL responses. In a related study, Kang et al., (1999) demonstrated that HIV-1 gag-env VLPs resulted in high CTL activity. Therefore, HIV-1 gag and env genes have been identified as strong candidates for use in an HIV/AIDS vaccine system and are therefore the genes of interest for this experiment.

In this study, the viral vector selected for HIV-1 gene expression was an attenuated recombinant vesicular stomatitis virus (VSV), a member of the Rhadoboviridae family. VSV infects cattle, horses, and swine. In humans, VSV infection is asymptomatic or may result in mild flu-like symptoms such as fever and malaise. VSV has a negative-sense RNA genome containing 11,161 nucleotides, which encode five major viral structural proteins, the nucleocapsid protein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G), and the large protein (L). VSV presents an attractive viral vector system due to its wide host range, rapid replication processes, and mild pathogenicity in humans. Kim and Kang (2007) found that mutations introduced into the M gene of VSV resulted in an attenuated virus that exhibited reduced inhibitory effects on host cell gene expression and in significantly less cytopathic effects within infected cell lines, finding allowing for the production of avirulent and temperature-sensitive VSV mutants.

Perhaps the most valuable trait of VSV is its existence in two serotypes, Indiana (VSV\textregistered{Ind}) and New Jersey (VSV\textregistered{NJ}). Both serotypes have the capacity to stably express foreign genes and exhibit no cross-neutralization, revealing the potential for usage of both VSV serotypes in a prime and boost method of vaccine delivery.

In order for the recombinant VSV vector to be used in an effective therapeutic HIV vaccine, the VSV vector system must be capable of stably expressing the HIV genes carried, an issue appraised in this study. In this research, the expression of HIV-1 gag and env genes in VSV\textregistered{Ind}(GML), an avirulent and temperature-sensitive mutant vector, was evaluated. High levels of HIV-1 gag and env gene expression by VSV\textregistered{Ind}(GML) would indicate the potential use of this viral vector system to activate CTLs and therefore confer immunity against infection through a therapeutic HIV vaccine. In this study, experimental procedures included the construction of the recombinant VSV\textregistered{Ind}(GML) vector, the recovery of the recombinant VSV\textregistered{Ind}(GML) mutant containing HIV-1 gag and env genes by reverse genetics, and the quantitation of levels of protein expression in cells infected with the recombinant VSV\textregistered{Ind}(GML) containing gag and env genes.

**Hypothesis**

It was hypothesized that the recombinant VSV\textregistered{Ind}(GML) vector would successfully express HIV-1 gag and env genes based on the characterization of the recombinant VSV as a tolerant viral vector that has already been identified as having the acute ability to effectively express a wide range of foreign genes. Quantitating the expression levels of HIV-1 Gag and Env proteins through the VSV\textregistered{Ind}(GML) vector system will indicate the potential for application of a recombinant VSV mutant as a major player in prospective therapeutic HIV vaccine development.

**Experimental Methods**

(1) **Plasmid Construction**

Independent construction of the vector was not performed as the recombinant VSV\textregistered{Ind}(GML) HIV-1gag-env vector was provided for this experiment by the Kang Laboratory. However, an explanation of the method of recombinant vector construction is needed in order to understand the results from
this study. VSV\textsubscript{ind}(GML), an avirulent and temperature-sensitive mutant, was produced in the Kang Laboratory through the introduction of mutations at G21E, M51R, and L111A. The \textit{gag} gene was fused with the \textit{env} gene containing a 171 nucleotide insertion that coded for T cell epitopes. The HIV-1\textit{ gag-env} hybrid gene was inserted into the VSV\textsubscript{ind}(GML) genome between the \textit{G} and \textit{L} genes by restriction enzyme \textit{Mlu1}.

(2) Cell culturing

Baby Hamster Kidney21 (BHK21) cells were used to amplify recombinant VSV\textsubscript{ind}(GML)\textit{-HIV-1 gag-env}. The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 5.0% fetal bovine serum (Gibco BRL), 100 \(\mu\)g/mL penicillin, 100 \(\mu\)g/mL streptomycin, 100 \(\mu\)g/mL kanamycin, and 2 mM L-Glutamine. The cells were incubated in a 5.0% CO\textsubscript{2} incubator at 37 °C for 17 hours and passaged when 100% confluent.

(3) Transformation of \textit{E. coli} DH5\textalpha

Competent Cell Preparation

\textit{E. coli} DH5\textalpha colonies were streaked on a Super Optimal Broth (SOB) agar media without antibiotics and incubated overnight at 37 °C. Five isolated colonies were transferred into 100 mL of SOB liquid media without antibiotics and grown using a shaking incubator set at 210 rpm at 37 °C. Cells were recovered by centrifugation at 4500 rpm for 4 °C for 10 minutes.

(4) DNA extraction by maxi-preparation

\textit{E. coli} DH5\textalpha colonies transformed with plasmids \textit{N}, \textit{P}, \textit{L}, and VSV\textsubscript{ind}(GML)\textit{-HIV-1 gag-env} were screened for successful recombination. Positive clones were confirmed using restriction enzyme mapping with digestion by enzyme \textit{Mlu1}. Three transformed colonies for each plasmid were subcultured individually in 2 mL of LB with ampicillin and shaken at 210 rpm at 37 °C for 17 hours. The subcultures were recovered by centrifugation at 5000 rpm at 4 °C for 10 minutes. The plasmid DNAs were purified and amplified to large quantities according to the MAXIprep protocol using Solution 1 [50 mM glucose, 25 mM Tris HCl (pH 8.0), 10 mM EDTA (pH 8.0) with the addition of lysozyme [1 mg/1 mL], Solution II [1% SDS, 0.2 M NaOH], and Solution III [7.5 M ammonium acetate]\textsuperscript{14}. DNA gel electrophoresis was performed to run extracted plasmid DNA alongside control plasmid DNA to verify successful extraction from bacterial cultures.

(5) Recovery of VSV\textsubscript{ind}(GML)\textit{-HIV-1 gag-env} by reverse genetics

Transfection was performed using BHK\textsubscript{T7} cells and Lipofectamine 2000 (Invitrogen) according to the manufacturer’s specifications in order to recover the recombinant VSV\textsubscript{ind}(GML)\textit{-HIV-1 gag-env} by the VSV recovery protocol\textsuperscript{12}. Confluent BHK\textsubscript{T7} cells were transfected with 19.4 \(\mu\)g of plasmid containing VSV\textsubscript{ind}(GML)\textit{-HIV-1 gag-env} and co-transfected with 10 \(\mu\)g of pKS-HN, and 5 \(\mu\)g of pKS-HL plasmid DNA. VSV-specific RNA polymerase was expressed through transcription of \textit{N}, \textit{P} and \textit{L} plasmid DNA by T7 RNA polymerase and \textit{N}, \textit{P}, and \textit{L} mRNAs were translated by Internal Ribosome Entry Sites (IRES). VSV\textsubscript{ind}(GML)\textit{-HIV-1 gag-env} was then transcribed and replicated by the VSV-specific RNA polymerase (\textit{N}, \textit{P}, \textit{L}), trimmed by HDV ribozyme, and assembled as the recombinant virus. Cells were incubated at 31 °C in a 5% CO\textsubscript{2} incubator because recombinant VSV\textsubscript{ind}(GML)\textit{-HIV-1 gag-env} is a temperature-sensitive mutant. The culture media was harvested when 80% of cells showed cytopathic effects (CPE), observed 96 hours post-transfection.
(6) Purification and determination of viral titer by plaque assay

Recovered virus, VSV<sub>Ind</sub>(GML)HIV-1gag-env, was purified three times by plaque picking with BHK21 cells. A plaque assay was then performed for viral titration in order to attain the viral titer. Recovered VSV<sub>Ind</sub>(GML)HIV-1gag-env was first diluted 10<sup>-4</sup>, 10<sup>-5</sup> and 10<sup>-6</sup> times. A 6-well plate of confluent Vero cells was inoculated with 100 μL of each dilution and incubated at 37 °C for 17 hours. Resulting plaque numbers were counted and calculations were performed to determine the virus titer, as shown in Table 1. The viral titer was then used to perform an infection of BHK21 cells at a multiplicity of infection (MOI) of 0.1 in order to propagate the recovered VSV<sub>Ind</sub>(GML)HIV-1gag-env and attain a viral stock. A second plaque assay titration was performed following the same procedure in order to determine the viral titer of the viral stock for infection at an MOI of 6 to prepare the cell lysate for SDS-PAGE and Western blot analysis.

(7) SDS-PAGE and Western blot analysis

Infections at an MOI of 6 of confluent BHK21 cells were performed with VSV<sub>Ind</sub>(GML)HIV-1gag-env and VSV<sub>Ind</sub>(GML) without an insert as a negative control. Infected cells were incubated at 31 °C and 37 °C, the permissible and semi-permissible growth conditions respectively for VSV<sub>Ind</sub>(GML) temperature-sensitive mutants. The protein in the cell lysate obtained from these infections was quantitated by UV spectrophotometry. The infected cell lysates were used to perform an SDS-PAGE followed by Western blot analysis. Blotting was performed using TBS-T buffer (0.1% Tween). Expression of VSV proteins, Gag and Env, were detected by Western blot analysis. For detection of VSV protein expression, a primary rabbit antibody of anti-VSV anti-serum was used with a secondary anti-IgG from rabbit. Detection of Gag expression was performed with a primary rabbit antibody, anti-p24 anti-serum, and a secondary anti-IgG from rabbit. Detection of Env was then performed with primary goat antibody, anti-gp120, and a secondary anti-IgG from goat.

Results

(1) Gel electrophoresis of extracted DNA by maxi-preparation

Results shown are from DNA gel electrophoresis with DNA plasmids N, P, and L (Fig. 1) and recombinant vector, VSV<sub>Ind</sub>(GML)HIV-1gag-env (Fig. 2). Samples of extracted DNA plasmids were electrophoresed alongside corresponding stock control plasmids. Electrophoresis results showed that the fragment sizes of extracted plasmids were identical to those of the marker plasmids, indicating the successful extraction of these plasmids from bacterial cultures.
(2) Recovery of VSV\textsubscript{Ind}(GML)HIV-1\textit{gag-env} by reverse genetics

Bacteriophage T7 RNA polymerase is constitutively expressed in BHK\textsubscript{T7} cells and this RNA polymerase transcribes the transfected pVSV\textsubscript{Ind}(GML)HIV-1\textit{gag-env} cDNA genome and co-transfected N, P and L plasmids. An Internal Ribosome Entry Site (IRES) allowed translation of VSV N, P, and L proteins, the VSV-specific RNA polymerase. After initial transcription of the recombinant VSV\textsubscript{Ind}(GML)HIV-1\textit{gag-env} genome by T7 RNA polymerase, it was replicated and transcribed by VSV-specific RNA polymerase (N, P, and L proteins) and the recombinant VSV\textsubscript{Ind}(GML)HIV-1\textit{gag-env} was assembled and released as a recombinant virus. Culture media containing the recombinant virus was harvested after observation of CPE in 80% of transfected cells 96 hrs post-transfection.

(3) Determination of viral titer

Sample calculations for the determination of the viral titer for the recovered virus and the viral stock are shown in Table 1 for plasmids diluted 10\textsuperscript{6} times. The first row shows the values and calculations performed to determine the viral titer of the recovered virus so that recovered virus could be propagated in cells through an infection at MOI 0.1 to obtain a viral stock. The second row shows the values and calculations performed to determine the titer of the viral stock in order to perform an infection at MOI 6 to obtain cell lysate for Western blot analysis.

(4) Quantitation of levels of protein expression by SDS-PAGE and Western blot analysis

Virus-infected cell lysates were analyzed by SDS-PAGE followed by Western blot analysis to quantify the levels of viral protein expression. This was performed using cell lysate prepared from infections with VSV\textsubscript{Ind}(GML) containing no insert and VSV\textsubscript{Ind}(GML)HIV-1\textit{gag-env} at 31 °C and 37 °C respectively. Expression of HIV-1 Gag

![Figure 4. Recovery of VSV\textsubscript{Ind}(GML)HIV-1\textit{gag-env} using reverse genetics. BHK\textsubscript{T7} cells are transfected with pVSV\textsubscript{Ind}(GML)HIV-1\textit{gag-env} and co-transfected with pN, pP, and pL. Culture media was harvested from transfected cells after observation of 80% CPE (16).](image-url)
and Env proteins are marked by arrows in the following figures. Fig. 5 shows expression levels of the full VSV protein, which served as a positive control. Lysates from BHK21 cells infected VSV<sub>ind</sub> containing no genetic inserts served as a vehicle control for detection of the expression of full VSV protein, HIV-1 Gag55, and HIV-1 Env. Fig. 6 shows the expression of HIV-1 Gag55 and Fig. 7 shows the expression of HIV-1 Env.

**Discussion**

In summary of the experimental procedures performed, the major steps of this study comprised of vector construction with the insertion of an HIV-1 gag-env hybrid gene into the VSV<sub>ind</sub>(GML) genome, recovery of the recombinant virus by reverse genetics, and quantitation of levels of protein expression in infected cell-lines. The procedures performed were successful in obtaining the results needed to make conclusive evaluations of the VSV<sub>ind</sub>(GML) vector system.

Results indicate that the VSV<sub>ind</sub>(GML) vector system allowed for high levels of expression of HIV-1 gag and env genes. This finding reveals the potential to use a genetically modified recombinant VSV as a universal vector for the development of recombinant virus vaccines. The VSV<sub>ind</sub>(GML) mutant vector is thus an attractive candidate for the viral vector of a therapeutic HIV vaccine. These major findings are concluded through the following analysis of results:

1. Gel electrophoresis of DNA extracted through maximal preparation confirmed successful extraction of pN, pP, pL, and VSV<sub>ind</sub>(GML)HIV-1 gag-env from E. coli DH5α bacterial cultures. This was established from the results of gel electrophoresis shown in Figures 2 and 3, which depict identical fragment sizes for corresponding control and experimental plasmids runs.

2. Recovery of VSV<sub>ind</sub>(GML)HIV-1 gag-env using reverse genetics was accomplished with the harvest of culture media containing the recombinant virus after observation of CPE in 80% of transfected BHK<sub>21</sub> cells 96 hours post-transfection. A longer incubation time was required for infections with VSV<sub>ind</sub>(GML) than infections performed with native VSV<sub>ind</sub> because VSV<sub>ind</sub>(GML) is an attenuated, avirulent mutant.

3. The results acquired from SDS-page and Western blot analysis indicated that VSV<sub>ind</sub>(GML) containing no insert and VSV<sub>ind</sub>(GML)HIV-1 gag-env both showed the same levels of VSV<sub>ind</sub> protein expression at both permissive and semi-permissive temperatures. This indicated that the stability of VSV<sub>ind</sub> was not compromised by HIV-1 gene insertion. The high levels of expression of both HIV-1 Gag and Env proteins in VSV<sub>ind</sub>(GML)HIV-1 gag-env demonstrates that the VSV<sub>ind</sub>(GML) vector system is capable of stably expressing HIV genes gag and
env, revealing the potential of VSV\textsubscript{Ind}(GML) for use in a recombinant virus vaccine system.

Similar results were shown in a study performed by Rose et al. (2000), who demonstrated the use of a recombinant VSV to effectively express HIV-1 genes through the sequential boosting of mice with recombinant VSV vectors encoding the HIV-1 Env protein. They observed a great increase in the production of HIV-specific neutralizing antibodies. CTL activation was not evaluated, but, a parallel study performed by Egan et al. (2004), demonstrated a significant increase in CTL response in macaques vaccinated with recombinant VSV-based vectors expressing HIV-1 Env and simian-human immunodeficiency virus (SIV) Gag proteins\textsuperscript{16}. These studies’ observations support this experiment’s results, demonstrating the recombinant VSV vector’s potential to express and process HIV-1 Gag and Env proteins. Such results are also supported by the findings of Wu, Kim, & Kang (2009) who, in addition to highlighting the capability of the VSV\textsubscript{Ind}(GML) vector system to express HIV-1 genes, found that its counterpart, the VSV\textsubscript{Ind} serotype, also possessed the ability to express HIV-1 gene, env (gp160). This is a finding that can be used in conjunction with this study’s results to confirm the capability of both serotypes of VSV to express HIV-1 genes\textsuperscript{8}. This suggests the potential of employing both VSV vector systems in the development of priming and boosting vaccines, using the VSV expression system to activate CD8 positive CTLs.

To conclude, VSV\textsubscript{Ind}(GML)HIV-1gag-env is capable of expressing HIV-1 Gag and Env proteins at both permissive and semi-permissive temperatures. For application to a recombinant virus vaccine system, it is desirable to develop a RNA virus vector capable of accommodating large foreign genes, allowing high levels of foreign gene expression. Therefore, this study’s results reveal the potential use of VSV\textsubscript{Ind}(GML) as an efficient viral vector system for a candidate therapeutic HIV vaccine to clear chronic HIV infection. This presents optimistic prospects for the development of a vaccine system that can substitute the high prices of anti-retroviral therapeutic drugs with a cost-effective immunotherapy that will help clear infection from millions of people diagnosed with HIV.

Future Work

Current research focuses on the application of recombinant virus vaccine systems for the treatment of other infectious diseases such as the hepatitis C virus and even bacterial diseases such as tuberculosis. The results from this experiment suggest that additional projected studies of protective immune responses in animal models and the evaluation of the side-effects of recombinant virus vaccine systems may be beneficial in expanding our knowledge of the immunological mechanisms underlying viral infections and subsequent development of more effective and economic anti-viral therapies.

Acknowledgements

I thank Dr. Chil-Yong Kang and Dr. Sally Wu for supervising this project. This research opportunity was offered by the Scholar’s Electives Program and coordinated by Kathy Boon. Additionally, the Kang Laboratory at Siebens-Drake Research Institute hosted this project with the support of the Canadian Institutes of Health Research, NHRDP of Health Canada, IRAP of the National Research Council Canada, Sumagen Co. Ltd., and Western University.

References


