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Development of HIV-1 subtype C *Gag* based DNA vaccine construct

Research Article

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Abbreviations: cytomegalovirus, (CMV); immediate early, (IE); kilodalton, (kD); phosphate buffered saline, (PBS); room temperature, (RT); virus like particles, (VLPs)

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Summary

Recently, the success of genetic immunization as a novel means to induce protective immunity has been demonstrated. DNA vaccines mimic antigen presentation closely to the natural history of viral infection. This is particularly relevant in infectious diseases where-in cell mediated immunity plays a larger role in protection, such as HIV-1 infection. In this paper we present the work done towards development of a *gag* based DNA immunogen for local circulating HIV-1 subtype C viruses in India. *Gag* gene was cloned under the control of CMV promoter in a mammalian expression plasmid vector. The other main features of the expression cassette in the construct pJWgagprotease49587 are bovine growth hormone polyadenylation signal and a *t-PA* leader signal. The construct was confirmed for expression *in vitro* by various means, p24 antigen capture assay, immunoblotting and electron microscopy. The TEM studies on transiently transfected COS-7 cells showed the presence of virus like particles (VLPs) as a consequence of gene expression from the construct pJWgagprotease49587. This finding is the first report of VLPs for a subtype C based *gag* construct. We expect that this construct will be able to prime a good immune response when used in *in-vivo* mice studies owing to the formation of virus like particles from the construct *in vitro.*

I. Introduction

Of the various infectious diseases that are responsible for morbidity and mortality, AIDS is deemed to be the fourth-biggest killer. HIV/AIDS is not a homogenous pandemic. Human immunodeficiency virus HIV-1, the causative organism has remained particularly elusive owing to the sheer diversity of viral evolution. The varied subtypes and more varied distribution have had profound impacts on the strategies being devised to control the spread of HIV infection. Most of the world's HIV infection is located in the developing world. Of these, most infections occur within the non-B HIV subtypes. Subtype C accounts for more than 50% of overall infections worldwide (Tatt et al, 2001). It is needed to direct resources towards the research of virus evolution, pathogenesis, treatment and preventive/therapeutic vaccines of different HIV-1 clades.

The need for developing a potent immunogen from the local circulating types is becoming more and more apparent with the evidence of differences in the rates of transmission and severity of disease among different clades. The current rapid spread of subtype C viruses has raised questions about the role of subtypes on disease progression and transmission. The presence of three NF-kB binding sites in subtype C viruses suggests that they might have a replication advantage. In India, infection rate at 0.8% of the total adult population is still low, but due to large population it transforms into large numbers. The use of existing therapies in the developing world is limited owing to their high cost (Dayton et al, 2000).

Nucleic acid vaccination offers a simple and effective means of immunization. DNA plasmids encoding foreign proteins have been successfully administered either by direct intramuscular injection or with various adjuvants and excipients, and by biolistic immunization.

DNA vaccines have several distinct advantages, presentation of target protein by MHC-I and MHC-II pathways, synthesis of immunogen in their native with appropriate post-translational modifications, ease in manufacturing process and greater shelf life of DNA as compared to proteins. This approach is particularly relevant to tumor antigens and viral immunogens.

Gag gene is one of the most conserved regions of HIV-1 genome and hence it is a good target for cross clade immune responses. It encodes for group antigen core protein. 1.5 Kb gene gives rise to a 55-kilodalton (kD) Gag precursor protein, also called pr55, which is expressed from the unspliced viral mRNA and later processed into the respective p24, p17, p6 proteins by the viral encoded protease. In studies with HIV infected individuals, HEPS and LTNPs, helper and cytotoxic responses to gag epitopes have been defined (Gotch et al, 1990; Jhonson et al, 1991; Kalams et al, 1999).

Plasmids used as DNA vaccines, in general contain a strong eukaryotic promoter, such as cytomegalovirus (CMV) immediate early (IE) (Chapman et al, 1991) and polyadenylation signal from bovine growth hormone, which increases expression. Immune response elicited by DNA vaccination depends on route of immunization, it is largely Th1 type, and this is particularly beneficial since Th1 type of immune response has been implicated in control of HIV infection. In this study we present the construction of a gag based plasmid immunogen in a mammalian expression vector and verification of its expression.

II. Materials and methods

A. Plasmid, cells and reagents

The vector used in the study, pJW4304, was a kind gift from Dr J. I. Mullins, University of Washington, Seattle, USA. COS-7 cells for *in vitro* expression studies were obtained from NCCS, Pune, India.

B. Cloning of *gag* gene into pJW4304

The integrated HIV-1 proviral DNA from PBMCs of HIV infected asymptomatic individual (Disease stage: A1, CD4 counts: 534/µl) was taken as a template for PCR and a 4.35 kb gag-pol (nt139 – nt4495) product was obtained by a set of nested PCRs using forward primers, MSF12: 5'AAATCTCTAGCAGTGGCGCCCGAACAG3' [1-27], GagFP01: 5'TTTGACTAGCGGAGGCTAGCAGGAGAGAG ATGGGT3' [139-173] and reverse primers PolRP06: 5'AAAACCATCCATTAGCTCTCCTTGAAACAT3' [4471-4500], PolRP01: 5'CATCCATTAGCTCTCCTTGAAACATAC ATA 3' [4466-4495]. The amplification profile was as follows: denaturation [at 92°C for 15sec], annealing (at 52°C for 30 sec] and extension [at 68°C for 4min] for 25 cycles followed by final extension for 7 minutes at 68°C. The amplification product was cloned into TA cloning pGEMT easy vector (Promega, USA) as per the manufacturer's instructions (**Figure 1A**). The construct was verified in pGEMTeasy by PCR and restriction digestions. The construct was double digested with Nhe1 and BamH1 enzymes resulting in the release of a 2.3kb Gag-protease fragment. This fragment was cloned into mammalian expression vector, pJW4304, by directional cohesive ends ligation (**Figure 2A**). The presence of insert in the plasmid pJWgagprotease-49587 was confirmed by PCR for gag and protease genes, restriction digestions and DNA sequencing.

C. *In vitro* expression studies

COS-7 cells were transfected using lipofectin reagent (Life technologies) according to the manufacturer's instructions. Briefly, 5µg plasmid DNA was constituted with lipofectin reagent at a concentration of 10µg/ml in DMEM (without FCS and antibiotics) and overlaid on 40-50% confluent COS-7 cells. The cells were incubated with the transfection mix for 6-8 hrs at 37°C, 5% CO_2 and then fresh medium was supplemented (DMEM 10% FCS, 2mM glutamine and antibiotics). The cells and supernatants were harvested at different time points 24, 36, 48, 72 and 96 hrs and stored at -20°C for further evaluation. COS-7 cells transfected with vector pJW4304 alone and the plasmid containing envelope gp120 gene, pJWSK3, (Arora et al, 2001) comprised the controls in the study.

D. p24 antigen capture ELISA

The supernatants were checked for presence of p24 antigen by p24 antigen capture ELISA (Innogenetics Belgium) performed as per the manufacturer's instructions. Briefly, 100µl of sample and the standard (provided in the kit) were aliqoted into the wells coated with anti p24 monoclonal antibody and incubated at 37°C in a humidified chamber for an hour. The wells were then washed thoroughly five times and tapped to remove traces of wash buffer. Thereafter 100µl of HRP conjugated anti p24 monoclonal antibody was added to the wells and the plate was incubated for an hour at 37°C followed by 5X washing again. In the next step 100µl of substrate solution was added to the wells and incubated in dark at room temperature for 30 minutes. 50µl of stop solution was added to the wells after the incubation and absorbance was recorded at 450nm. Standard curve was plotted for the absorbance recorded for standard provided in the kit and concentration of the samples was determined from the curve. The negative controls included untransfected cells and cells transfected with vector alone (pJW4304) and mock positive (pJWSK3) control.

E. Western blot analysis

The transfected cell lysates were run on a denaturing SDS PAGE and transferred onto nitrocellulose membrane by semidry transfer method. The blot was blocked with 2.5% non-fat dry milk in Tris buffered saline pH 7.4 for two hours at room temperature (RT) and was washed thrice in TTBS (Tween-Tris buffered saline). Immunoblotting was carried out by incubating with HIV-1 positive human serum (at a dilution of 1:50) at RT for 1hr. After washing thrice the blot was returned for incubation with alkaline phosphatase conjugated goat anti-human IgG antibody for an hour at RT. Thereafter, it was washed thrice and the substrate (BCIP-NBT solution) was added. The reaction was then stopped by washing in double distilled water.

G. Electron microscopy of transfected COS-7 cells

Transmission electron microscopy was performed with transfected cells as described earlier (Gheysen et al, 1989) with minor modifications. Briefly, transfected cells were scraped off, washed in phosphate buffered saline (PBS pH 7.4) and then fixed in 1% glutaraldehyde solution for two hours on ice. Thereafter, the cells were washed with PBS thrice and postfixed with 1 % osmium tetroxide in PBS for two hours. After washing with PBS and then with distilled water, the fixed cells were stained with 1% uranyl acetate in 20% acetone for 30 min. The cells were dehydrated by treatment with acetone and cleared with toluene. Thereafter, infiltration was done with toluene araldite mixture first at room temperature and then at 50°C temperature. The

sample was embedded in epoxy resin, sectioned and viewed under TEM (transmission electron microscope).

***Footnote:** The HIV-1 subytpe C strain 49587 used in this study is from a hemophilic patient who got infected through blood tranfusion in 1989 in India. (patient id# 49587). The PBMC sample was collected in the year 1997 from the northern part of India. The Genbank accession number isAF533140.

III. Results

A. Construction of pJWgagprotease49587

In order to clone *gag-protease* genes of HIV 1 subtype C, a complete *gag-pol* clone was generated in pGEM-Teasy by PCR based TA cloning (**Figure 1A**). A 4.3 Kb PCR product was generated by a nested set of primers MSF12 and Pol RP06 and GagFP01 and PolRPO01 (**Figure 1B**). This product was ligated to pGEM-Teasy vector and the recombinant was screened on the basis of blue white colony selection. The 4.3Kb *gag-pol* insert was confirmed by *Eco*R1 digestion of the plasmid that releases the complete gene fragment (**Figure 1C**). PCR products from different regions of the construct, 1.5-Kb *gag* and 3-Kb *pol* confirmed the presence of insert, *gag-pol,* in the clone pGEMTgag-pol. (**Figure 1D**).

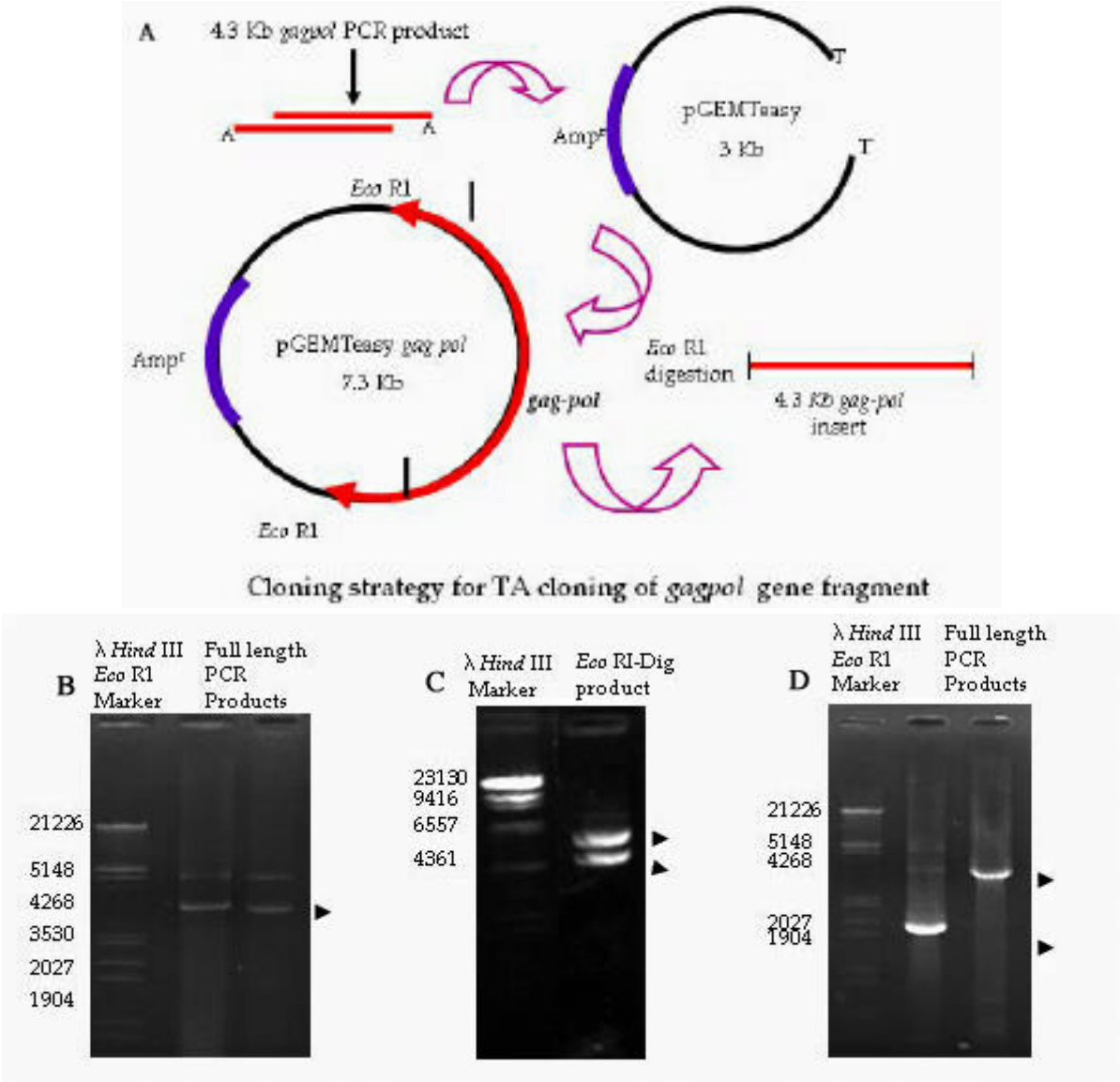


Figure 1A Cloning strategy for TA cloning of gagpol gene fragment. A 4.3Kb fragment generated by nested PCR was cloned into pGEM-Teasy vector resulting in a recombinant molecule pGEM-Teasy gag-pol (7.3Kb). **B** Agarose gel picture showing, 4.3 Kb gag-pol PCR product generated by nested set of PCR with λ *Hind III Eco R1* DNA molecular weight marker in the adjacent lane. **C** Agarose gel picture showing the release of 4.3 Kb gag-pol fragment from pGEMT-easy gag-pol upon EcoR1 digestion. **D** Complete *gag* (1.5 Kb) and *pol* (3.1 Kb) PCR amplification products from the pGEMTeasy gag-pol.

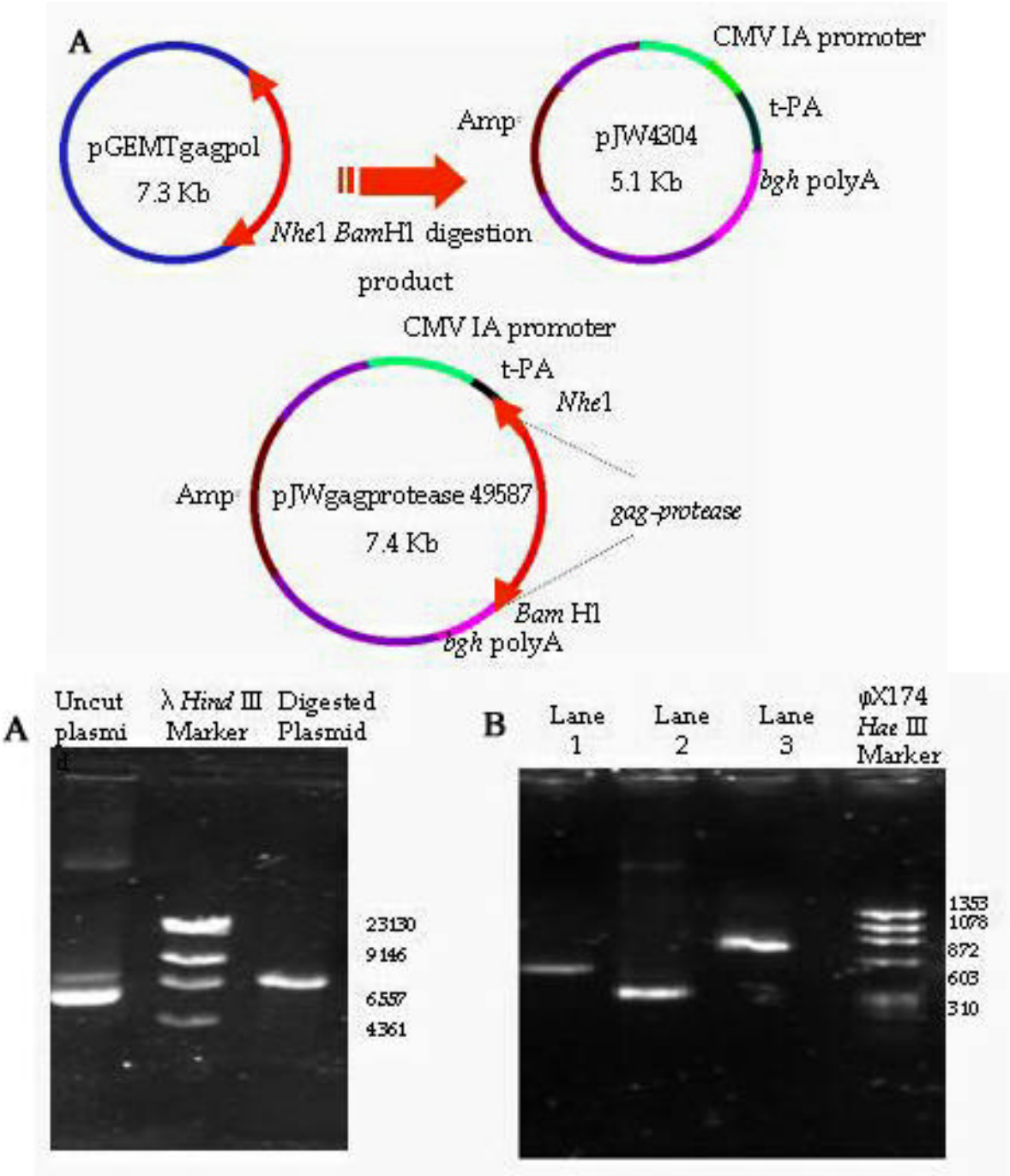


Figure 2A Strategy for cloning gag-protease fragment into eukaryotic expression vector pJW4304. A double digestion of pGEMT-easy gag-pol with restriction endonucleases *Nhe1* and *BamH1* releases a 2.3 Kb fragment containing the gag and protease genes. This fragment was then ligated into pJW4304 by cohesive ends ligation. **B** Agarose gel picture showing 7.4 kb linearised plasmid pJWgagprotease-49587 along with λ *Hind III* molecular weight marker. **C** Agarose gel picture showing PCR amplification products for sub-genomic fragments of *gag* & complete *protease* genes. The amplification products for *gag* are 492 bp and 711bp respectively in lanes 1 and 3. The *protease* gene fragment represented by 290bp PCR product is depicted in lane2.

From this clone the fragment containing *gag-protease* gene was extracted by double digestion with *Nhe*1 and *Bam*H1, and ligated into the expression vector pJW4304 (**Figure 2A**). The recombinant clone obtained was confirmed for the presence of required gene fragment by various digestions and PCR amplification products for *gag* and *protease* genes (**Figures 2B, C**). The right orientation of the insert in the clone was confirmed by *Pst*1 digestion, which released a 750 bp product as it should in case of correct orientation of the cloned gene. Further confirmation of the cloned gag-protease gene that it belonged to HIV-1 subtype C *gag* and *protease* regions, was obtained with sequencing using primer walking strategy. (GenBank Accession no: AF533140) (data not shown).

B. p24 Antigen Capture ELISA

The amount of protein secreted in the medium by the transfected COS-7 cells was assessed by p24 antigen capture ELISA. p24 antigen was detectable at 24-hrs post-transfection and showed a gradual increase in levels until 48 hrs and thereafter a decline was observed. Such an observation is typical of protein expression in transiently transfected cells. The negative controls included in the study were untransfected cells and cells transfected with vector pJW4304 (without any insert) and mock positive control pJWSK3 (envelope plasmid). None of the control supernates showed any reactivity in the assay. Up to 110-pg/ml protein was detected in the supernates (**Figure 3A**).

C. Immunoblotting

The transfection cell lysates were run on SDS PAGE and transferred onto nitro cellulose membrane for immunoblotting using HIV positive sera as a source of polyclonal antibodies to HIV proteins. The 24-kilodalton band representing gag p24 was detected in the 24 and 48 hrs cell lysates indicating that the 55kilodalton-Gag precursor was being cloven into respective products. The negative controls and mock positive cell lysates did not show any such band (**Figure 3B**).

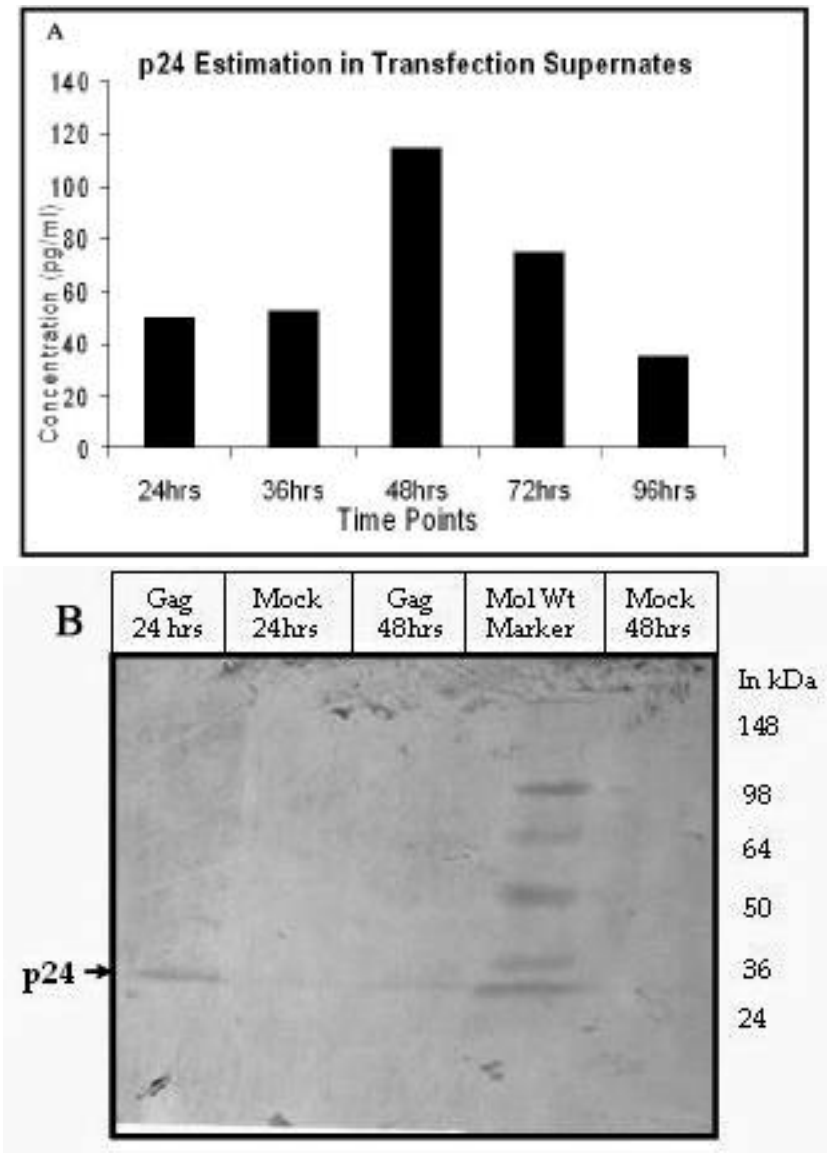
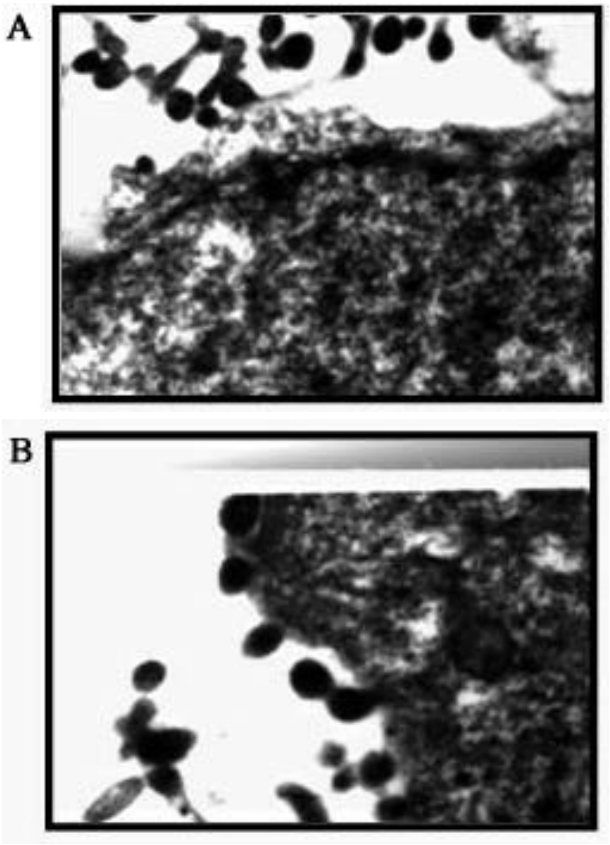


Figure 3A p24 estimation in transfection supernatants during a time course experiment by p24 antigen capture ELISA plotted for the various dilutions of reference standard p24, provided in the kit (Innogenetics Belgium). Maximum amount of p24 was detected at 48 hrs post-transfection, thereafter the amount of p24 in the medium declined. **B** Immunoblotting was done with pJWgagprotease-49587 (denoted as gag in the figure) and pJW4304 (denoted as Mock in the figure) transfected cell lysates. SDS PAGE was run and proteins were transferred onto nitrocellulose membrane by semi dry transfer method. The blot was probed with HIV positive human sera (ID no: 757) as a source of polyclonal antibodies to various HIV proteins. In the figure, immunoblot shows 24Kd band representing Gag protein (p24) in the 24 hrs and 48hrs transfected cell lysates. The untransfected cell lysates did not show the presence of any HIV-1 specific band.



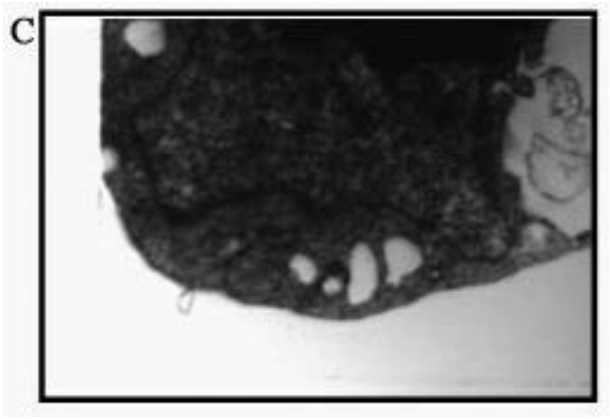


Figure 4A, **B**. Transmission electron micrographs of COS-7 cells transfected with pJWgagprotease-49587. TEM was done with cells harvested at 24 and 48 hr post-transfection. Budding protrusions from the cell membrane are seen representing VLPs. Average particle size was determined to be in the range of 140 to 160 nm. (magnification (a) 23,000 X and (b) 18,000X) **C** Transmission electron micrograph of pJW4304 transfected COS-7 cells as control. No virus like particles are visible either on the surface or outside the cell membrane. (magnification 14,000 X)

D. Electron microscopy of transfected cells

In transmission electron micrographs numerous virus like particles (VLPs) were seen budding out of the cell membrane and lying outside the membrane in the intercellular spaces. The morphology of these particles corresponded to that of a pr55 VLP. These VLPs were observed in pJWGagprotease-49587 transfected COS-7 cells at 24 and 48 hr post transfection. The average size of the particle was determined to be 140 nm-160 nm (Fig 4. a & b). Such particles were not seen in normal untransfected cells and cells transfected with vector alone (pJW4304) and untransfected cells (**Figure 4C**).

IV. Discussion

Both structural (env, gag, pol) and nonstructural genes (rev, nef) have been targeted as candidate immunogens for elicitation of effective immune response to HIV-1. The surface e*nvelope* glycoprotein gp 120 has been extensively studied as a potential target for HIV-1 vaccine development. The variable nature of envelope, particularly V3 loop, has proven to be a major hurdle in elicitation of cross-clade responses. The importance of targeting envelope gp120 remains, as it is the first HIV-1 protein that is encountered by the immune system in the natural history of pathogenesis. In our laboratory we have developed an envelope based DNA vaccine construct and tested in mice model for immunogenecity (Arora et al, 2001). However in view of the importance of cross-clade broad immune response we sought to develop a gag based immunogen. Cross clade CTL responses have been demonstrated within the gag region in studies with infected individuals (McAdams et al, 1998). The importance of gag-based responses is also derived from the studies showing the co-relation of Th responses to gag p24 in patients with non-progressive state of HIV-1 infection (Rosenberg et al, 1997). It has also been shown that an early HAART rescues helper responses to gag p24, which enables the immune system to keep the virus under control. The distribution of CTL and Th epitopes in HIV-1 gag reveals presence of 81 CTL and 27 Th epitopes in gag p24, 35 CTL and 5 Th epitopes in p17 and 2 CTL and 6 Th respectively in the nucleocapsid (p15) regions. These data from the HIV molecular immunology database clearly show the relevance of targeting gag gene of HIV-1(Los Alamos Immunology Database).

In challenge studies with chimeric virus SHIV 83.6 in primates, SIV *gag* constructs have been used to immunize the animals. The tetramer binding assays showed that the presence of large frequency of precursor CTL against HIV-1 *gag* gene was coincident with the clearance of challenge virus. These studies underline the importance of targeting *gag* gene in a vaccine construct

Considering all these factors we set out to design an effective immunogen based on Indian clade C HIV-1 viruses. Our objective was to develop a DNA vaccine construct from local circulating subtype C virus strain, which is the most predominant subtype prevalent in the Indian population. In our strategy for construction of *gag-protease* plasmid we have cloned the gene fragment in conjunction with the *t-PA* leader signal sequence present in the vector pJW4304. The use of *t-PA* leader sequence is

known to have positive effects on expression of Envelope and Gag proteins as demonstrated in other studies. Use of *t-PA* leader signal has shown better immune responses as compared to cytoplasmic targeting of *gag* gene (Qui et al, 2000).

The viral *protease* gene was cloned along with *gag* gene in order to provide the native protease for proper processing of *gag* gene products from the precursor pr55 protein into p17, p24, p6, p7, and p2. This gene encodes for an aspartyl protease enzyme that recognizes and cleaves the gag precursor pr55 into respective gene products, p17, p24, p15, p6 and p2. *Protease* gene is expressed as -1 frameshift from the *gag* open reading frame in the HIV-1 genome. This frameshift occurs once in twenty times during translation of gag-pol open reading frame. In our cloning strategy the frameshift site was preserved hence allowing the synthesis of both the proteins as in their native infection process of mammalian cells. Another obstacle in over-expression of *protease* is that it leads to complete processing of gag particles which abolishes VLP formation in cells, hence we considered it beneficial to keep the original frame shift site in the gag protease construct pJWgagprotease-49587.

In *in-vitro* expression studies, we detected upto 110pg/ml of secreted antigen in transfected COS-7 cell supernatants (**Figure 3A**). In addition, a 24-kilodalton band representing p24 gag (**Figure 3B**) was observed on immunoblotting. This shows that the viral protease expressed from the construct has been successful in processing the pr55 precursor gag protein into respective products. We also observed formation of virus like-particles (VLPs) at 24 and 48 hrs post transfection in COS 7 cells (**Figures 4A, B**). These VLPs were in the size range of 120-160 nm. This is the first report of production of virus like-particles from an HIV-1 subtype C based construct. The production of VLPs from the vaccine construct adds the advantage of particulate antigen to priming with DNA based immunogen.

The earlier studies with *gag* gene examined the particle formation in various expression systems and evaluated the probable use as particulate antigen. Antigens in particulate conformation have been shown to be highly immunogenic in mammals. Expression of *gag* gene alone has shown that self-assembly of p55 molecules triggers the formation of pseudovirions or VLPs (Nermut et al, 1998). Virus like particles have been described in studies with baculovirus, vaccinia, yeast and mammalian expression systems (Gheysen et al, 1989; Haffar et al, 1990; Wagner et al, 1992). A study by Wagner and coworkers examined particle formation by *gag* constructs in various expression systems (Wagner et al, 1992). Budding of 100-160 nm pr55 core particles resembling immature virions was observed in eukaryotic systems. They proposed that empty immature gag particles would represent a safe non-infectious and attractive immunogen. Thereafter several studies have been published demonstrating the immunogenicity of the virus like particles. Long-lived cellular immune responses have been elicited upon administration of VLP formulations in murine and monkey models (Paliard et al, 2000; Rovinski et al, 1995; Wagner et al, 1998). The hybrid HIV-1 p17/p24:Ty-VLP vaccine module that has gone into phase I trials has demonstrated the ability of inducing both cellular and humoral immune responses to p17 and p24 proteins. VLPs have also been designed for inclusion of principal neutralizing domain of gp120 and other regions of envelope proteins for successful elicitation of both neutralizing humoral immune response and cytotoxic T cell response (Brand et al, 1995; Buonangaro et al, 2002).

In a recent study immunogenicity of virus like particles consisting of gag, protease and envelope from clade B HIV-1 in rhesus macaques was assessed. In this study three different forms of antigens were delivered, purified VLPs, recombinant DNA and canarypox vectors engineered to express VLPs. It was found that nucleic acid vaccination capable of producing VLPs was more efficient in priming cell-mediated immune responses (Montefiori et al, 2001). It is understood that in order to induce $CD8^+$ T cell memory, the antigen needs to be presented via the MHC class I pathway. It has also been demonstrated that cross presentation of HIV-1 virus like particles by dendritic cells can lead to efficient priming of CTL responses (Bachman et al, 1996). These studies have implicated that recruiting dendritic cells for antigen presentation of exogenous virus like particles in a DNA vaccine module is an added advantage. In view of the above discussion, it can be expected that the production of virus like-particles from our DNA vaccine construct, pJWgagprotease-49587, would have a combined effect of DNA vaccine and particulate antigen in one module.

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