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DNA Vaccination for the induction of immune responses against HIV-1 subtype C envelope gene in mice

Research Article

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Abbreviations: bovine growth hormone, (BGH); cytomegalovirus, (CMV); cytotoxic T lymphocyte, (CTL); *envelope*, (*env*); human immunodeficiency virus, (HIV); Joint United Nations Program on HIV/AIDS, (UNAIDS); lactate dehydrogenase, (LDH); National Centre for Cell Science, (NCCS); peripheral blood mononuclear cells, (PBMCs); stimulation index, (SI); tetramethylbenzidine dihydrochloride,, (TMB); tissue plasminogen activator, (*tPA*); World Health Organization, (WHO)

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Summary

Most human immunodeficiency virus (HIV) DNA vaccines currently being developed are based on clade B strains of HIV-1, which are found predominantly in North America and Europe. Since in India, subtype C is the predominant strain of HIV-1, it is imperative that a vaccine based on the local circulating subtype should be designed. Two DNA constructs encoding HIV-1 *envelope glycoprotein* (*gp120*) obtained from HIV-1 subtype C primary isolates were used for immunising mice. Mice immunised intramuscularly with these constructs produced low levels of antibodies against Gp120. However, these animals showed MHC class I restricted cytotoxic T lymphocyte (CTL) activity against homologous (subtype C) as well as heterologous (subtype B) peptide pulsed target cells thus demonstrating cross clade reactivity. In addition, *in vitro* lymphocyte proliferation and Th1 cytokine response to HIV antigen stimulus was seen with high levels of IFN-γ and IL-2 but undetectable IL-4 and IL-5 production. These findings indicate that these constructs have possible value as potent vaccines. Their further characterization in non-human primate models is warranted.

I. Introduction

The need for an effective vaccine against human immunodeficiency virus type 1 (HIV-1) has never been greater. It has been estimated by the Joint United Nations Program on HIV/AIDS (UNAIDS) and World Health Organization (WHO), that more than 36 million people are currently infected with HIV worldwide. Although the epidemic was initially recognized in industrialized countries, it is spreading most rapidly in the developing world with more than 95% of new infections occurring in these countries. Although new antiretroviral drugs have been able to prolong life of HIV infected individuals (Moreno et al, 2000), the high cost of such therapy puts it beyond reach for most of the world (Berger, 1996; Hogg et al, 1998). In addition, these drugs are partially successful because of side effects associated with prolonged use and the development of viral resistance to these drugs (Max et al, 2000; Servais et al, 2001). Therefore, a safe and effective HIV preventive vaccine is urgently needed to bring the HIV/AIDS epidemic under control. Historically, live-attenuated vaccines have been able to elicit a complete and long-lasting immunity (Melnick et al, 1994), but an attenuated HIV that is still able to replicate raises obvious safety concerns (Baba et al, 1995, 1999; Desrosiers, 1998; Johnson, 1999). DNA vaccines serve as an alternative to the live attenuated virus. DNA vaccines have been effective in generating immune

response and protection in a wide variety of preclinical models of viral, bacterial and parasitic infections, and cancer (Hoffman et al, 1997; Inchauspe et al, 1997; Lozes et al, 1997). Intramuscular vaccination with plasmids expressing HIV-1 genes have been shown to generate specific CTL and helper T cells and antibodies in mice and nonhuman primates (Wang et al, 1993, Otten et al, 2000; Cherpelis et al, 2001). A therapeutic phase 1 trial on humans with such constructs induced a good safety profile and also demonstrated an immunologic potentiation (Ugen et al, 1998, Boyer et al, 2000; Mac Gregor et al, 2000). Virtually all the HIV DNA vaccines being developed are based on clade B strains of HIV (which are found predominantly in North America and Europe).

The *envelope* (*env*) gene of HIV-1 encodes for a precursor glycoprotein 160 (Gp160) which is cloven by a cellular protease to surface glycoprotein Gp120 and a transmembrane glycoprotein Gp41. Gp120 and Gp41, are assembled into a trimeric complex that mediates virus entry into target cells (Weiss et al, 1990; Poumbourios et al, 1995). Earlier we have demonstrated that HIV-1 (subtype B) *gp160, tat, rev* based DNA constructs resulted in induction of cell mediated immune response but antibody response was low and transient in mice (Arora and Seth, submitted). HIV-1 *env* glycoprotein has been shown to contain some immunomodulatory sequences in gp41, which are known to downregulate antibody responses (Haynes et al, 1993). Also it is known that replacing the signal sequence of HIV-1 env gene with signal sequence from human tissue plasminogen activator protein (tPA), yields better expression of env gene (Golden et al, 1998). In addition, signal sequence of HIV-1 envelope glycoprotein inhibits the folding of HIV-1 Env protein (Li et al, 2000) which may result in poor antibody response. Therefore, we cloned HIV-1 *gp120* gene from two Indian isolates of HIV-1 into mammalian expression vector in frame with tPA signal sequence and studied its expression *in vitro* in HeLa cells. Mice were intramuscularly immunized with these constructs. DNA immunization resulted in induction of low antibody response thus indicating that replacing leader sequence of HIV-1 *env* with that of tPA leader sequence and removal of gp41 did not result in increased antibody response. However, we observed specific and cross-reactive CTL and helper T lymphocyte responses. CTL response is known to play a major role in controlling primary viremia (Koup et al, 1994), and also long term non-progressors have been shown to have strong CTL response (Harrer et al, 1996). The findings indicate that these constructs have possible value as potent vaccines. Their further characterization in non-human primate models is warranted.

II. Results

A. Construction of vectors expressing HIV-1 Gp120

HIV-1 *gp120* was PCR amplified using a nested PCR approach. DNA isolated from peripheral blood mononuclear cells (PBMCs) of two HIV-1 infected individuals (referred to as Ch18 and SK3, respectively) were used as template DNA. Several studies have demonstrated that the HIV-1 *gp120* gene with its natural signal sequence expressed in any prokaryotic or eukaryotic expression systems showed extremely low levels of synthesis and secretion (Murphy et al, 1993; Li et al, 1994; Golden et al, 1998). Therefore, a sense primer was designed internal to the signal sequence of HIV-1 *env* gene and *Nhe* I restriction site was introduced in the primer so that *gp120* gene could be cloned in frame with human tissue plasminogen activator (*tPA*) signal sequence in pJW4304 mammalian expression vector. The *gp120* from two isolates was cloned into pJW4304 mammalian expression vector in frame with tPA leader sequence to generate plasmids pJWCh18 and pJWSK3.

B. Subtyping of HIV-1 strains

The subtypes of the infecting viruses were characterized by sequencing of the C2-V3-C3 region and also by heteroduplex mobility assay (HMA). Sequencing revealed that both the patients (Ch18 and SK3) were infected with HIV-1 subtype C (**Table 1**). Heteroduplex mobility assay showed that the HIV-1 *gp120* insert in clone

Table 1. Sequence of V3 region of *gp120* gene of Ch18 and SK3 isolates.

Ch 18 (Sequence map points : 795-1182)

5'-AGATCTGAAAATCTGACAAACAATGTCAAAACAATA
ATAGTGCACCTTAATGAATCTGTAGAAATTGTGTGTAC
AAGACCCAACAATAATACAAGAAGAAGTATAAGAATA
GGACCAGGACAAGTATTCTATGCAAATAATGACATAA
TAGGAGACATAAGACAAGCACATTGCAACATTAGTAA
GGATGTCTGGAACAGTACTTTACAAAAGGTAGGTAAAA
AATTAAAAGAACACTTCCCTAATAAAACAATAACATTT
GAACCACACTCAGGAGGAGATCTAGAAATTACAACAC
ATAGTTTTAATTGTAGAGGAGAATTTTTCTATTGCAATA
CATCAGGGCTGTTTAAAAGTAACTTTAATGATACAGAA
GGTAATTCAACTT-3'

SK 3 (sequence map points : 794-1183)

5'-TTAGATCTGAAAATCTGACAAACAATGTCAAAACAA
TAATAGTGCACCTTAATGAATCTGTAGAAATTGTGTGT
ACAAGACCCAACAATAATACAAGAAGAAGTATAAGAA
TAGGACCAGGACTAGTTTTCTATGCAAATAATGACATA
ATAGGAGACATAAGACAAGCACATTGCAACATTAGTA
AGGATGTCTGGAACAGTACTTTACAAAAGGTAGGTAAA
AAATTAAAAGAACACTTCCCTAAAAAAACAATAACATT
TGAACCACACTCAGGGGGAGATCTAGAAATTACAACA
CATAGTTTTAATTGTAGAGGAGAATTTTTCTATTGCAAT
ACATCGGGGCTGTTTAAAAGTAACTTTAATGAAACAGA
AGGTAATTCAACTT- 3

pJWCh18 was most closely related to subtype C3 and that in clone pJWSK3 was most closely related to subtype C2 (**Figures 1a &b**).

C. *In vitro* expression

HeLa cells were transiently transfected with pJW4304, pJWCh18 or pJWSK3. A time course experiment was performed and cells culture supernatant and cell lysate of transiently transfected HeLa cells were analyzed for expression of HIV-1 *gp120* by pJWCh18 and pJWSK3 vectors. Western blot analysis using HIV-1 positive human polyclonal serum demonstrated that HIV-1 Gp120 was present in cell lysate at 48-60 hrs post transfection. Gp120 was also secreted into the culture supernatant derived from pJWCh18 and pJWSK3 transfected cells (results shown for 72-hrs post transfection). Cell lysate and cell culture supernatant obtained from cells transfected with pJW4304 did not react with HIV-1 positive human polyclonal serum (**Figure 2**).

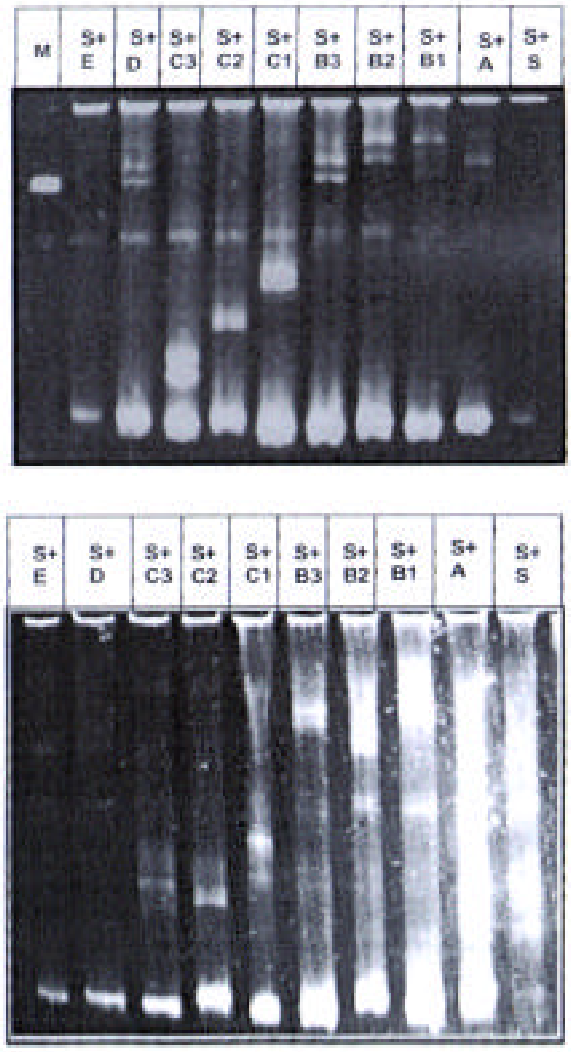

Figure 1. Heteroduplex mobility analysis of HIV-1 gp120 genes cloned in **a**) pJWCh18 and **b)** pJWSK3.

D. Animal immunisation

Balb/c mice injected with pJW4304, pJWCh18 and pJWSK3 DNA constructs thrice at 4-week intervals were used to study both humoral and cell mediated immune response generated as a result of DNA immunisations.

E. Lymphocyte proliferation response

Splenocytes from mice immunised with three doses of pJWCh18 showed stimulation index (SI) of 11.4 and 6.12 on stimulation with homologous peptide, pep10 and heterologous peptide pep09 respectively. Similarly, SI of 8.3 and 6.2 was observed when splenocytes from mice immunised with 3 doses of pJWSK3 were stimulated with pep10 and pep09 respectively. SI of 7.6 and 7.1 was observed on *in vitro* stimulation with recombinant Gp120 of splenocytes from mice immunized with pJWCh18 and pJWSK3, respectively. Splenocytes from pJW4304 (vector without the insert) immunised mice showed S.I. of <2 on stimulation with either peptide or recombinant Gp120 (**Figure 3**).

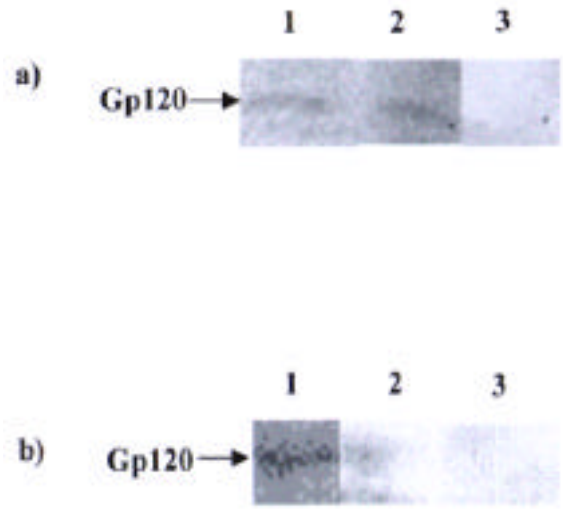

Figure 2. (**a**) Western Blot analysis of HeLa cell lysates transfected with pJWCh18 (lane 1); pJWSK3 (lane 2) and pJW4304 (lane 3) 60 hrs post transfection. (**b**) Western Blot analysis of cell culture supernatant from HeLa cells transfected with pJWCh18 (lane 1); pJWSK3 (lane 2) and pJW4304 (lane 3) 72 hrs post transfection. Western Blot analysis was performed using HIV-1 positive human polyclonal serum

E. Lymphocyte proliferation response

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F. Cytokine production by splenocytes

Splenocytes from mice immunised with 3 doses of either pJWCh18 or pJWSK3 constructs, on stimulation with homologous (pep10) or heterologous (pep09) peptide or recombinant Gp120, produced significantly high levels of IFN-γ and IL -2 in culture supernatants (**Figure 4**).

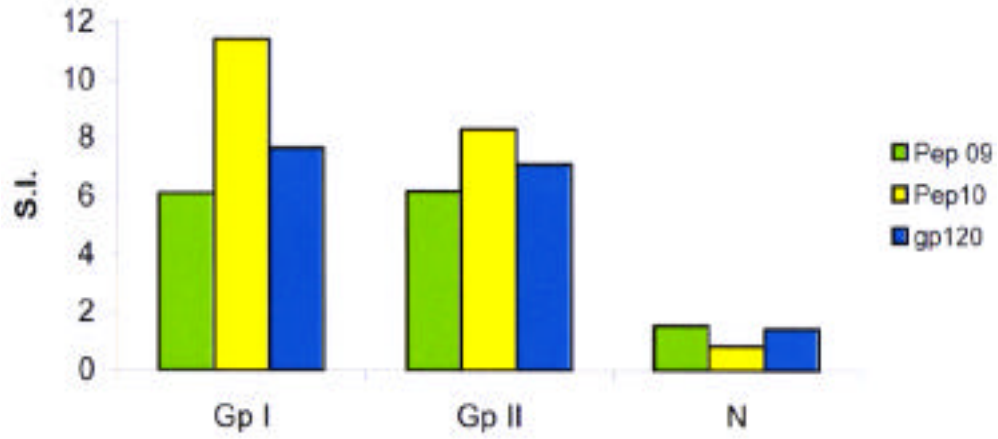

Figure 3. *In vitro* T Cell proliferative responses to homologous and heterologous peptides (pep09 and pep10) and recombinant gp120 from HIV-1 gp120 DNA immunised mice. Mice were immunized 3 times with 100µg of either of the constructs pJWCh18 (Gp I) or pJWSK3 (Gp II) at 4 week intervals, while control mice (N) were injected with the vector pJW4304 alone (without the insert). Splenocyte cultures were incubated with 20µg/ml of the stimulating peptides and 1µg/ml of recombinant gp120 and harvested 72 hrs later.

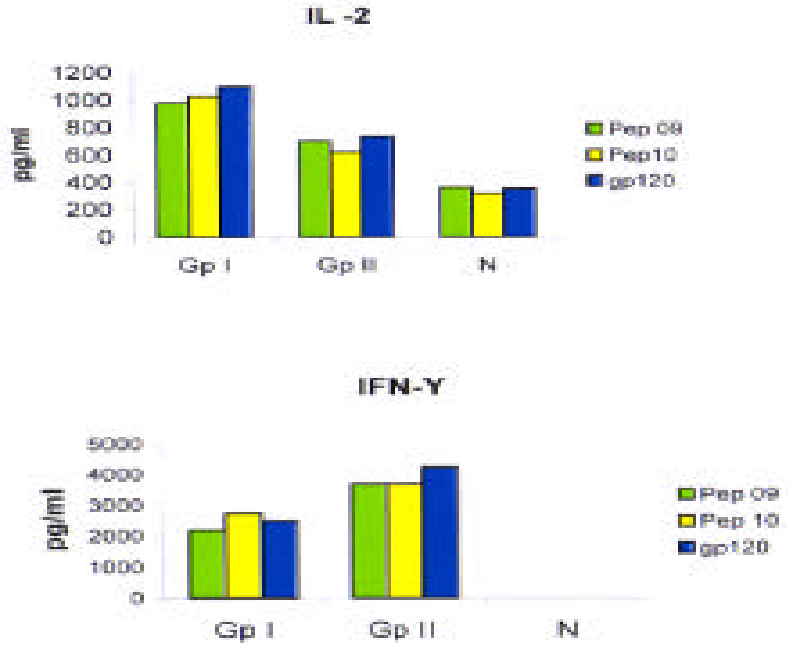

Figure 4. *In vitro* cytokine secretions from splenocyte culture from mice immunised intramuscularly with three doses of 100µg of pJWCh18 (Gp I), pJWSK3 (Gp II) and vector alone, pJW4304 (N). Supernatants were tested for cytokines (IL-2, IFN-γ, IL-4 and IL-5) on the third day of culture with homologous (pep10), heterologous (pep09) peptides, or recombinant gp120. IL-4 and IL-5 levels were undetectable (data not shown).

However, IL-4 and IL-5 levels were undetectable in these stimulated cultures (data not shown). Cultures of splenocytes from mice immunised with the vector alone (without insert) showed undetectable to low levels of all the cytokines.

G. Cytotoxic T lymphocyte (CTL) responses

Cytotoxic-T cell activity was measured in splenocytes harvested from the immunised mice 4 weeks after the third dose. Effector cells from pJWCh18 immunised mice demonstrated comparable CTL activity against homologous (pep10) or heterologous (pep09) peptide pulsed P815 target cells. The lysis remained within a range of 33%-48% at various graded effector to target ratios. Whereas, effector cells from pJWSK3 immunised mice demonstrated an increase in CTL activity (12.7%-51%) with corresponding increase in effector to target ratio. The lysis was greater for homologous peptide (pep10) pulsed target cells as compared to heterologous peptide (pep09) pulsed target cells. Unstimulated target cells (N) were taken as control targets and effector cells from mice immunized with either pJWCh18 or pJWSK3 showed negligible CTL activity against unstimulated target cells (**Figure 5**). Effector cells from control animals (immunised with the vector DNA alone) demonstrated negligible CTL activity against either pep09 or pep10 pulsed target cells (data not shown).

H. Humoral immune response

Low levels of HIV-1 specific antibody response was detected in mice immunised with three doses of either pJWCh18 or pJWSK3 against recombinant Gp120 protein coated plates in ELISA and not against peptide pep10 (data shown for pJWCh18 only). Sera obtained from animals immunised with three doses of recombinant vaccinia virus expressing gp120 gene of HIV-1 subtype B (vPE8) reacted strongly with recombinant Gp120 as well as pep10 and served as a positive control (**Figure 6**).

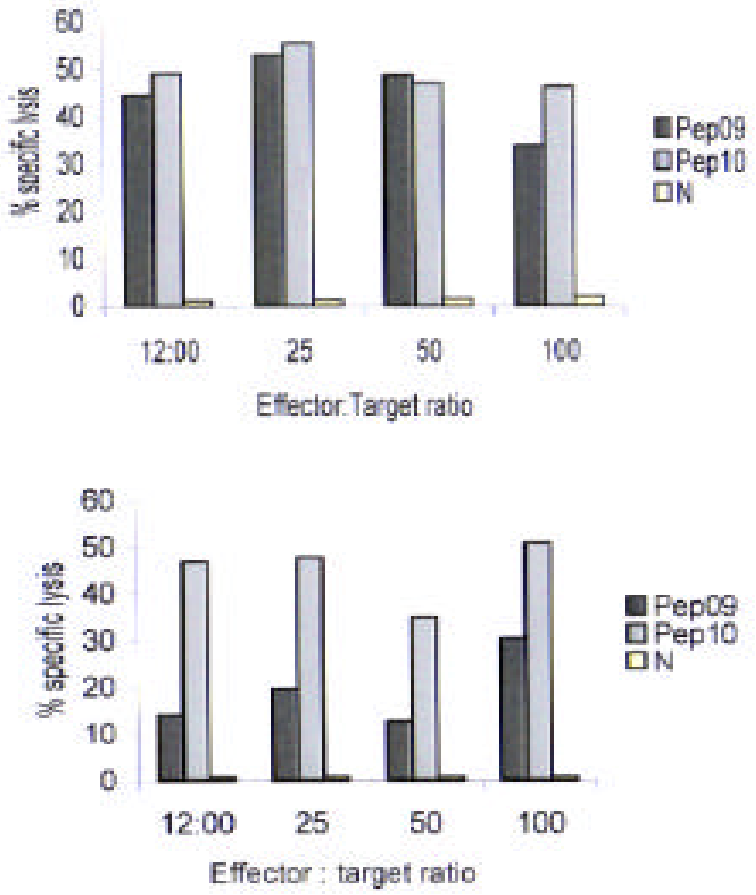

Figure 5. Induction of cytotoxic T lymphocytes in mice immunised with pJWCh18 (panel **a**) and pJWSK3 (panel **b**) plasmid DNA. Female Balb/c mice (H-2^d) (4-6 weeks old) were injected intramuscularly three times at four-week interval with 100µg of either construct. Spleen cells were used as effector cells against mouse mastocytoma cells, P815, as target cells that were stimulated with 20µg/ml of either pep10 (homologous peptide) or pep09 (heterologous peptide). Unstimulated target cells (N) were taken as control targets.

III. Discussion

Present study demonstrated Gp120 specific CTL responses against both subtype C (pep10) and subtype B (pep09) stimulated target cells, using effector cells from mice immunised with pJWCh18 and pJWSK3 showing that CTLs are capable of crossreacting with non-C clade target cells. Effector cells from either pJWCh18 or pJWSK3 immunised mice showed comparable CTL activity against both homologous peptide, pep10 and heterologous peptide, pep09 pulsed target cells (**Figure 5**). Effector cells from mice immunised with pJWSK3 also demonstrated CTL activity against homologous peptide, pep10 and heterologous peptide, pep09 pulsed target cells. However, percent specific lysis was greater for homologous peptide pulsed target cells as compared to heterologous peptide pulsed target cells (**Figure 5**). Broadly reactive cross clade CTL activity is highly desirable as the extent of HIV-1 sequence diversity affects the efficacy of an HIV vaccine. Cross-clade HIV-1 specific cytotoxic T lymphocyte responses have been demonstrated in HIV-1 infected individuals. Betts et al, (1997) demonstrated that CTLs from HIV-1 C clade infected individuals kill autologous targets expressing HIV-1 clade B derived Gag, Pol or Env. The induction of CTLs in Balb/c mice after immunization with envelope DNA constructs has been reported earlier (Wang et al, 1995; Kim et al, 1997). Shiver et al, (1995) showed that mice immunized with *gp120* DNA exhibited Gp120 specific MHC class I restricted CTL activities utilizing V3 peptide-sensitized P815 cells as targets.

Several groups have independently reported successful results generating HIV-1 specific antibody and CTL responses in mice and non-human primates (Fuller et al, 1994; Lu et al, 1995; Shiver et al, 1995). Increasing evidence indicate a protective role for cytotoxic T lymphocytes in the host defence against HIV infection. Much of the evidence for the role of HIV specific CTLs in controlling HIV infection has come from the observation of CTL activity in HIV infected people at different stages of the disease. An HIV-1 specific CTL response has been detected at the time of primary infection and is maintained vigorously in long-term non progressors (Harrer et al, 1996). The initial viremia is controlled by CTL response as it is found that the appearance of the latter occurs just as the viremia falls, although antibodies with the capacity to neutralise the virus are rarely detected at this stage (Koup et al, 1994). A decline of antiviral CTL is usually coincident with disease progression (Klein et al, 1995). This loss of CTL activity is secondary to the loss of CD4+ T cell numbers (Pantaleo et al, 1995; Fauci et al, 1996). CTL reactivity against HIV antigens has been detected in seronegative persons who had been exposed to the virus. These include a small number of commercial sex workers in Africa, sexual partners of infected persons, children born to infected mothers and health care workers exposed to infectious body fluids (Clerici et al, 1993, 1994; Fowke et al, 1996).

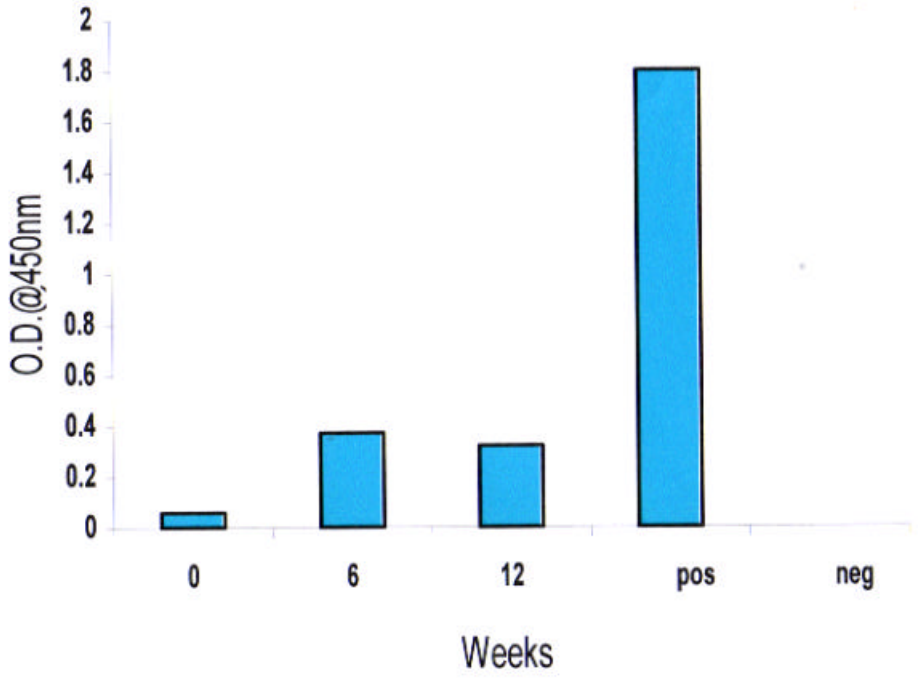

Figure 6. Antibody response to HIV-1 recombinant gp120 in the serum samples (1:10 serum dilution) collected 2 and 4 weeks after the second and third dose respectively in pJWCh18 immunised mice. Pos = positive, Neg= negative.

Memory T cell responses, as manifested by proliferation of antigen specific T cells and secretion of cytokines during *in vitro* culture of splenocytes have been shown by intramuscular immunisations with DNA plasmids encoding a variety of antigens such as influenza HA and NP, HIV Env, and Rev (Shiver et al, 1997). The present study demonstrated that the splenocytes from pJWCh18 and pJWSK3 immunised mice showed antigen specific proliferation in response to stimulation with peptide from homologous strain, (pep10) and to a lesser extent with a peptide from a heterologous strain (pep09). Thus indicating that helper T cell crossreactive lymphoproliferative immune responses were induced. The profile of cytokine secretion in response to antigen restimulation of spleen cells from immunised mice was indicative of a Th1 like helper T cell response i.e. high IL-2 and IFN-γ levels and negligible level of IL-4 and IL-5 levels. Similarly, Shiver et al, (1997) demonstrated *in vitro* proliferation and Th1 like cytokine secretion from various lymphoid sites from *gp120* (subtype B) DNA-immunised mice. Th1 cells are crucial to the containment of HIV. A steady shift from Th1 to Th2 has been reported in HIV infected individuals with disease progression (Clerici and Shearer, 1993). Th1 pattern has also been observed in response to a panel of HIV envelope peptides in a group of seronegative individuals who remain uninfected despite repeated exposures to HIV (Clerici et al, 1994).

In the present study direct inoculation with DNA construct (pJWCh18 or pJWSK3) encoding HIV-1 *gp120* gene induced low antibody response in mice as demonstrated by ELISA on sera obtained 2 - 4 weeks following primary and the booster doses. Similarly, low levels of ELISA antibodies have been detected in mice immunised with plasmids expressing Gp120 from BaL and JR-FL, which are prototypic monocyte/macrophage tropic virus strains of subtype B as compared to the antibody levels obtained on immunisation with DNA constructs expressing Gp120 from a T-cell line adapted virus (Richmond et al 1997). In the present study also primary isolates were used as the source of *gp120* gene for the preparation of DNA constructs.

The present study shows that DNA vaccines not only are potentially effective in generating Th1 response and CTL response but that these responses are also cross-reactive. Therefore, it is reasonable to design a vaccine capable of inducing cellular immune responses. Nonetheless, the studies in mouse model on potential HIV-1 vaccine strategies are limited to demonstrating immunogenicity only. Since these animals are neither susceptible to infection with HIV-1 nor the subsequent immunodeficiency, studies of Indian HIV-1 subtype C DNA vaccine preparations in non-human primate models should be pursued.

IV. Materials and Methods

A. Cells and reagents

HeLa and P815 (mouse mastocytoma cells) cell lines were purchased from National Centre for Cell Science (NCCS) Pune, India. P815 cells were maintained in DMEM and HeLa cells in MEM, supplemented with 10% fetal calf serum (FCS), antibiotics and glutamine. Murine IFN-γ, IL-2, IL-4 and IL-5 ELISA kits were purchased from Endogen Inc.Woburn, MA. HIV-1 gp120 V3 loop peptides, Pep 09 (RGPGRAFVTI-OH, aa313-322) corresponding to HIV-1 subtype B and Pep 10 (RIGPGQTFYATG-OH, aa 313-324) corresponding to HIV-1 subtype C were commercially synthesised for us by Commonwealth Biotechnologies Inc., Richmond, VA. Recombinant gp120 was provided by the NIH AIDS Research and Reference Reagent Program, Bethesda, MD). Plasmid vector pJW4304 was a kind gift from Dr. J.I. Mullins (University of Washington, Seattle, WA) and pCR-Script SK (+) cloning vector was purchased from Stratagene, LaJolla, CA. Plasmids were grown in DH5α strains of *Escherichia coli* (Life Technologies, Gaithesburg, MD), and purified using Wizard miniprep columns (Promega Corp, Madison, WI).

B. Animals

2-4 weeks old inbred female BALB/c mice, were purchased from National Central Laboratory Animal Sciences (NCLAS), Hyderabad, India.

C. DNA construction

PBMCs were separated from two asymptomatic HIV-1 seropositive individuals by Ficoll-Hypaque density gradient method. Genomic DNA was extracted using Qia amp blood kit (Qiagen Inc. Stanford, CA) following manufacturer's instructions. DNA encoding the gp120 region of the HIV-1 was molecularly cloned using a nested PCR approach. First, a 1531 bp fragment containing gp 120 gene was amplified using following primers:

Sense primer (E00) - 5' -TAGAAAGAGCAGAAGACAG TGGCAATGA-3' (-24-2)

Antisense primer (E75) - 5'-GCGCCCATAGTGCTT CCTGCTGCTCCC-3' (1507-1481)

This fragment was further amplified using a second set of primers to generate a 1436 bp gp120 fragment:

Sense primer (APpcr501)- 5'-GTCGCTCCGCTAGC TTGTGGGTCACAGTCTATTATGGGGTACC-3' (84-118)

Antisense primer (APpcr505)- 5'-GGTCGGATCC *tta*CTCCACCACTCTCCTTTTTGCC-3' (1469-1448)

Nhe I and *Bam*H I sites (underlined) were introduced in the sense and antisense primers, respectively in order to clone the gp120 sequence in frame with the *tPA* signal sequence in pJW4304. A stop codon (in lower case) was introduced preceding the *Bam*H I site in the antisense primer.

PCR amplified fragments were cloned directly into pCR-Script Amp SK (+) cloning vector following manufacturer's instructions. HIV-1 *gp120* from two isolates was then excised from pCR-Script by cleavage with *Nhe* I and *Bam*H I and cloned into mammalian expression vector pJW4304 to produce pJWCh18 and pJWSK3. DNA constructs pJWCh18 and pJWSK3, therefore expressed *gp120* (amplified from the PBMCs of two HIV infected individuals) under the control of cytomegalovirus (CMV)

immediate-early promoter and polyadenylation sequences from bovine growth hormone (BGH).

D. Subtyping

Heteroduplex mobility assay was performed using Heteroduplex Mobility Analysis HIV-1 *env* subtyping kit (Delwart et al, 1994) provided by the NIH AIDS Research and Reference Reagent Program, Bethesda, MD as per kit instructions. Briefly, a nested PCR approach was used to generate 0.7-kb *env* gene fragments. Initially, a 1436 bp-*gp120* fragment was amplified using outer primers APpcr501 and APpcr505. This amplified product was then used as a template DNA to amplify 0.7-kb *env* gene fragment using inner primers ES7 and ES8, which spans V3-V5 coding domain of *gp120.*

Sense primer (ES7) - 5' CTG TTA AAT GGC AGT CTA GC 3' (771-790)

Antisense primer (ES8) - 5' CAC TTC TCC AAT TGT CCC TCA 3' (1392-1372)

The same size fragments were also amplified from a series of plasmids containing HIV-1 *env* genes from different subtypes used as references. Heteroduplexes formed between the sample and the most closely related reference sequence exhibited the fastest mobility and thus indicated the likely subtype of that strain.

E. *In vitro* expression

In vitro expression of plasmids (pJWCh18 and pJWSK3) was tested in transiently transfected HeLa cells. Lipofectin (Life Technologies, Gaithersberg, USA) was used as a transfection reagent. At 48-72 hr post transfection, cell free supernatants were collected and cells were harvested by washing with PBS (pH7.2) and detaching with 0.1%EDTA. Expression was studied by western blot analysis of the transfected cells. HIV-1 positive human polyclonal serum was used as a source of antibody. Cells transfected with pJW4304 served as the controls.

F. DNA inoculation

A facilitated DNA immunisation protocol was followed which resulted in increased protein expression levels from plasmids delivered genes *in vivo* (Wang et al, 1993). Specifically, the quadriceps muscles of BALB/c mice (seven mice per group) were injected with 50µl of 0.5% bupivacaine hydrochloride and 0.1% methyl paraben (Sigma Chemicals Co., St. Louis, and CT) in normal saline using a 27-gauge needle. Forty-eight hrs later, 100µg of DNA construct was injected into the same region of the muscle as the bupivacaine injection. Mice were injected with DNA thrice at 4-week intervals. In a typical experiment, 7 mice in each of three groups were inoculated with DNA constructs including vector, pJW4304, alone (as control) or vector with gp20 insert from 2 different isolates of HIV-1 subtype C, pJWCh18 and pJWSK3. Blood was collected from each mouse for antibody assay just prior to immunisation, at the end of second and fourth week following first dose and then after 4 weeks of subsequent doses. For determination of cell mediated immune response, spleens from immunised mice four weeks after the final boost were aseptically removed and single cell suspensions were prepared. The cells from the same group of mice were pooled. RBC's were removed by treating the spleen cells with 0.9%NH_4Cl for 1 min at 37°C, followed by two washes in HBSS containing 2%FCS and resuspended at a concentration of $2x10^6$ cells/ml in RPMI 1640 with 10% FCS, antibiotics and glutamine.

G. Lymphocyte proliferation assay

One hundred microliters of the splenocyte suspension ($2x10^6$ cells/ml) was added to each well of a 96-well flat bottom tissue culture plate. Cells were stimulated in triplicate with V3 peptides (either pep09 or pep10) at a concentration of 20µg/ml or recombinant gp120 at a concentration of 1µg/ml. The cells were incubated at 37° C in 5% CO_2 for a total of 72 hrs. 16-18 hrs prior to harvesting one µCi of ^3H thymidine (Bhabha Atomic Research Centre, Mumbai, India) was added to each well. The cells were harvested and the amount of ^3H thymidine incorporated was measured in a 1211 Minibeta liquid scintillation counter (LKB - Wallac, Finland). Splenocytes from vector inoculated mice served as negative controls. Con A was used as a polyclonal stimulator positive control. Basal levels of ^3H-thymidine uptake by the splenocytes were obtained by culturing the cells in medium alone. Stimulation Index (SI) was calculated by the following formula:

$$SI = \frac{\text{mean c.p.m. Of } (^3H) \text{ thymidine incorporated in the presence of stimulated antigen}}{\text{mean c.p.m. of } (^3H) \text{thymidine incorporated in the unstimulated medium control cell cultures}}$$

S.I. greater than 3 was taken as a positive response.

H. Cytokine assay

Pooled splenocytes ($2x10^5$ cells) were cultured with 20µg/ml V3 peptides (pep 09 and pep10) or recombinant Gp120 at a concentration of 1µg/ml in a total volume of 200µl of RPMI 1640 containing 10%FCS in a 96 well tissue culture plate for 72 hrs at 37°C. The supernatants were harvested and assayed for the presence of IL-2, IFN-γ, IL-4 and IL-5 using commercially available ELISA kits as per manufacturer's instructions.

I. Cytotoxic T lymphocyte assay

Cytotoxic T lymphocyte (CTL) assays were performed as described earlier (Corr et al, 1996) with minor modifications. Briefly, stimulator cells were prepared by incubating $2x10^7$ normal syngeneic BALB/c splenocytes with 20µg/ml pep09 for 2 h at 37°C followed by irradiation (2500 rads) in a gamma cell irradiator (Gamma cell 3000 Elan, MDS Nordion). The effector cells were prepared by stimulating $5x10^7$ splenocytes from immunized mice with $1x10^7$ peptide pulsed and irradiated stimulator cells in 40 ml of RPMI 1640 containing 10% FCS, glutamine, antibiotics and $5x10^{-5}$ M 2-mercaptoethanol (Sigma Chemicals Co.,) and 10U/ml recombinant murine IL-2 (Roche Molecular Biochemicals, Manheim, Germany). The splenocytes were incubated at 37°C in 5% CO_2 for 5 days and then they were washed and used as effector cells.

Mouse mastocytoma cell line P815 was used as target cells. These cells were pulsed overnight with 20µg/ml of either pep 09 or pep10 peptides at 37°C in 5%CO_2. Thereafter, the target cells were resuspended in RPMI 1640 to a final concentration of $2x10^5$ cells/ml and 100µl of it were added to each well of a 96 well U bottom tissue culture plate. This was followed by addition of 100µl of effector cells to each well in triplicate at graded effector to target ratio. After a 4-hr incubation at 37°C in 5%CO_2, 50µl of supernatant was harvested from each well and transferred to a flat bottom 96 well plate. Lysis was measured by lactate dehydrogenase (LDH) release using the Cytotox 96-assay kit (Promega corp. Madison, WI). Controls were included in each plate for spontaneous LDH release from

target and effector cells. Percent cytotoxicity was calculated by the following formula as per manufacturer's instructions:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental- Effector spontaneous} - \text{Target spontaneous}}{\text{Target Maximum- Target Spontaneous}} \times 100$$

J. ELISA

High binding 96 well microtiter ELISA plates (Costar corp. Cambridge, USA) were coated with one µg of V3 peptide (pep 10) or recombinant Gp120 per well in 100 µl of carbonate- bicarbonate buffer (pH 9.6) at 37ºC for 1 hr followed by blocking with 0.8 %BSA in phosphate buffer saline (pH 7.2) at room temperature for 2 hrs. 1 in 10 dilution of mouse serum collected at various time intervals as indicated above, was allowed to react with the antigen at 37ºC for 60 minutes. Wells were washed 6 times with PBS - Tween 20 (pH-7.2) and incubated with goat anti mouse Ig conjugated with horseradish peroxidase (DAKO A/S, Denmark) for 30 min at 37ºC followed by washing. Then the substrate (H_2O_2) with 0.1 mg/ml of chromogen (3,3',5,5' tetramethylbenzidine dihydrochloride, (TMB), Sigma Chemicals Co., St. Louis, CT) in citrate acetate buffer (pH 5.6), was added to each well and incubated for 30 min at room temperature in dark. The reaction was stopped with the addition of 50µl of 1N H_2SO_4. The plate was read on a Labsystems Multiskan plus plate reader at OD 450nm.

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