STRUCTURE AND FUNCTION OF HIV-1 CRF01_AE ENVELOPE PROTEINS FROM BLOOD AND GENITAL FLUID ISOLATES

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Abstract. The recombinant envelope protein (gp120) of the human immunodeficiency virus type 1 (HIV-1) CRF01_AE *env* gene isolated from the corresponding blood (rgp120-F36PC) and genital fluid (rgp120-F36VC) specimens obtained from HIV infected individuals was successfully produced in both prokaryote and eukaryote cells. The yields of HIV-1 recombinant envelope proteins rgp120-F36PC and rgp120-F36VC produced in *E. coli* and in mammalian cells were 1.0 and 1.2, and 0.3 and 0.5 mg/ml, respectively. Antibody responses in mice immunized with rgp120-F36VC protein were not significantly higher than those with rgp120-F36PC protein. The level of antibody response in mice immunized with V3 deleted recombinant gp120 proteins from rgp120-F36VC and rgp120-F36PC was not significantly different from wild type rgp120 proteins. β-strands at the tip of the V3 loop of the HIV-1 envelope protein were predicted for the wild type genital fluid isolate but not for the wild type blood isolate. The replication capacity of both F36PC and F36VC was quite efficient. The infectivity assay of the epithelial cell line for pNL4-3/gp120F36VC was better than for pNL4-3/gp120F36PC. The extra β-strands in the V3 loop may be involved in cell tropism.

INTRODUCTION

HIV-1 causes a chronic infection in humans which results in defective cellular immunity secondary to CD4 lymphocyte destruction causing AIDS (Barre-Sinoussi *et al*, 1983; Gallo *et al*, 1984; Levy *et al*, 1984, 1998; Ostrowski, 2005). It has been two decades since the human immunodeficiency virus type 1 (HIV-1) was identified as the etiologic agent of acquired immunodeficiency syndrome, there is yet no effective vaccine against the virus. One of the biggest obstacles in developing an effective AIDS vaccine is the extreme difficulty in eliciting neutralizing antibodies (Nabs) that are broadly reactive against diverse HIV-1 strains (Burton *et al*, 1997; Parren *et al* 1997). The difficulty in eliciting Nabs against the virus comes from three major factors (Nyambi *et al*, 1998, 2000) the highly variable antigenic structure, the extensively glycosylated viral surface envelope glycoproteins, the conserved regions of the protein that binds cellular CD4 receptors and the coreceptors are obscured by variable loops (Thali *et al*, 1993). The poor immunogenic property of gp120 in eliciting Nabs against the virus is readily

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apparent from the analyses of the crystal structure of the gp120 core. A three dimensional model of the crystal structure shows a significant fraction of the exposed surface of the trimeric envelope glycoprotein on the virion is occupied by carbohydrate residues and a hypervariable region (Kwong *et al*, 2000). To elicit Nabs that have broad cross reactivity against a large number of genetically diverse HIV-1 isolates, a vaccine candidate has to be designed so the conserved regions of the gp120 are exposed as much as possible. A number of strategies have been proposed or are being explored to elicit broadly reactive Nabs, including the use of fusion intermediates, CD4 independent envelope glycoproteins, glycosylation site mutation, and variable loop-deleted envelope proteins (Wyatt *et al*, 1993; Barnett *et al*, 2001; Kim *et al*, 2003). The rationale behind these approaches is to expose conserved epitopes of the protein that are common to many HIV-1 isolates by either eliminating the shield (carbohydrates or variable loops) or by capturing various transitional states of the envelope.

Recently, different anatomical compartments have been the focus of attention as related to the evolution of HIV-1 in different environments. Several studies have identified the composition of the virus recovered from plasma and peripheral blood mononuclear cells (PBMC) (Simmonds *et al*, 1991; Livingstone *et al*, 1996) during HIV-1 infection. The virus was also found to have evolved into a variety of different viral sequences in the PBMC and mucosa (rectal and cervical)(Poss *et al*, 1995, 1998; Zhang *et al*, 2002). Genital secretions are the most common source of human immunodeficiency virus resulting in more than 90% of new infections transmitted heterosexually. Virus in the female genital tract may be important for both vertical (mother-to-child) and heterosexual (female-to-male) transmission. Previous studies examining portions of the HIV-1 genome have documented viral variants in the genital tract that differ from those in the blood (Delwart *et al*, 1998). Investigation of the genotypic and phenotypic features of HIV-1 isolates from the female genital tract is critical for the development of new therapeutic and preventive strategies to prevent heterosexual transmission.

Construction of recombinant protein from HIV-1 isolates was carried out. The protein produced was immunized into mice and the antibody response was studied. We then studied isolates with the V3 loop deleted in order to evaluate the effect of a change in structure on the immune response compared with the wild type. The three dimensional structure of each isolate was predicted and compared between the wild type and the type with the V3 deletion.

MATERIALS AND METHODS

Viruses

Two HIV-1 CRF01_AE isolates from the blood (F36PC) and genital fluid (F36VC) of an infected female (F36) were used in this study (Sutthent *et al*, 2001). Nucleotide sequences of the HIV-1 gp120 obtained from the blood and genital fluid were submitted to the Genbank with Genbank accession numbers of AY005172 and AY005173.

The biotypes of the F36PC and F36VC isolates were T tropic (syncytium inducing or SI) and M tropic (non-syncytium inducing or NSI), respectively. The coreceptor usages of F36PC and F36VC were CXCR4/CCR5 and CCR5, respectively (Sutthent *et al*, 2001).

The amino acid sequences of the F36PC and F36VC isolates were aligned using Lasergene Software version 5.0 (DNASTAR, WI) and sent for three-dimensional protein prediction via internet to the website http://

cubic.bioc.columbia.edu/predictprotein for analysis. The results were viewed using a freeware program called Spdbv. This program compares the homology of the protein under consideration and computes the molecular surface of proteins.

HIV-1 env (gp120) recombinant protein production

Recombinant protein expression in prokaryotic cells (Guzzman *et al*, 1995). HIV *env* gp120 expressed plasmid pBAD/His C was constructed. The HIV-1 *env* (gp120) gene was amplified by nested PCR with two pairs of primers: 1) upstream primer ENVA (5'-GGCTTAGGCATCCCTATTGCAGGAAGAAA-3') and downstream primer ENVK (5'-AGTAGTGGTGCAGATGAGTTTTCCAGAGC-3') followed by 2) upstream primer ENVAI1 (5'-ACTGCAGGGTACCATGGGGTTCCTGTATGG-3') and downstream primer ENVAI2 (5'-CTCGAGGAATTCTTCCTCCAGGTCTGAA-3'). The amplified product of the gp120, 1,260 base pairs in size, was cloned into a pDrive vector by a QIAGEN® PCR cloning kit (QIAGEN, Germany) and transformed into DH5α competent *E. coli* cells. Digested HIV-1 *env* (gp120) amplified DNA, *PstI/EcoRI* cut, from the pDrive containing the *env* gene, was cloned into the pBAD/His C vector and transformed into LMG194 competent *E. coli* cells.

The production of polyhistidine tagged protein from *E. coli* strain LMG 194 containing pBAD/His C-gp120 was induced with 0.2% arabinose and purified by a metal affinity column, MagneHis™ protein purification system (Promega, WI). The proteins were quantified using a BCA assay (Bio-Rad, CA). The protein was transferred to a nitrocellulose membrane (0.45 mm. SS) and a Western blot against pooled anti HIV-1 seropositive sera or anti-HIV-1 seronegative sera was carried out. The size of the gp120 recombinant protein was 51 kDa.

Recombinant protein expression in eukaryotic cells. Digested HIV-1 *env* (gp120) amplified DNA, *KpnI/XhoI* cut, from pDrive containing *env* gene, was cloned into a pSecTag2/Hygro C vector and transfected into a COS-7 cell line with Polyfect transfection reagent (QIAGEN, Germany) according to the manufacture's protocol. Hygromycin B (Boeringer Mannheim, Germany) at a concentration of 200 μg/ml, was used to select stable expression of pSecTag2/Hygro C-env. Recombinant protein from COS-7 was purified by a Ni-NTA spin column (QIAGEN, Germany).

HIV-1 *env* gene V3 deletion by overlapping extension (Kim *et al*, 2003)

Plasmid DNA from pDrive-gp120/F36PC and pDrive-gp120/F36VC was used as a template for amplification with primer ENVAI1 and gp1201RN (5'-CGGCCGACAATTGATTTCTACAGATTTATT-3') primers for the first round and gp120d2F (5'-GCCGGCTGTGAGATTAATGGAACAAAATGG-3') and ENVAI2 primers for the second round. The amplified products 700 and 900 base pairs in size were amplified with ENV AI1 and ENV AI2 primers, a hanging A was added on the terminal end of the nucleotide, and cloned into a QIAGEN® PCR cloning kit (QIAGEN, Germany). Then, Δ V3 gp120 DNA was cloned into pBADHis C and pSecHis/Hygro C in order to express Δ V3 gp120 recombinant protein in prokaryotic and eukaryotic cells.

Animal immunization

Mice were purchased from the National Laboratory Animal Center, Mahidol University, Thailand. Six to eight-week-old female BALB/c mice were immunized intraperitoneally (IP) with HIV-1 gp120 recombinant produced protein. The recombinant rgp120 proteins (wild type and Δ V3 gp120) from prokaryotic and eukaryotic cells at concentrations of 50, 100, 200 and 250 μg were

mixed with complete Freud's adjuvant at a ratio of 1:1 before immunization into the mice. Three mice were used for each concentration. The recombinant proteins produced from pBADHis C and pSecHis/Hygro C were used as control. One mouse was sacrificed to collect serum used as a control before injection. The recombinant proteins were boosted at 2 and 3 weeks after the first immunization. The mice were boosted with 50-250 μg of recombinant protein and mixed with incomplete Freud's adjuvant at a ratio of 1:1 before injection. After the last boost the mice were sacrificed and whole blood was collected for further study. Serum was prepared by centrifugation of clotted blood at 1,800*g* for 5 minutes, stored at -80ºC and utilized for anti-gp120 serology and a neutralization assay.

Anti-gp120 antibody response detection by enzyme-linked immunosorbent assay (ELISA) (Kim *et al*, 2003)

The presence of anti-gp120 specific antibody was determined by an ELISA assay; 5 μg of purified rgp120 or V3 deleted rgp120 protein coated with 50 μl of MagnaHis bead was used (Promega, WI). Mice sera was diluted (1:300) and added to the plate coated with rgp120 or V3 deleted gp120 protein. The plates were incubated at room temperature for 30 minutes with shaking. Horseradish peroxidase-labeled goat anti-mouse Ig (G+M+A) and TMB substrate were used for the ELISA. Reactions were stopped by adding 100 μl of 1N H_2SO_4. The absorbance was measured at 450 nm with an ELISA microplate reader. The cutoff value for absorbance was calculated by the formula: cutoff = 0.124 [(X+3SD) x 2], where X = mean of all negative sample absorbance +3 standard deviations) x 2. The ELISA index (EI = Absorbance / cutoff) is a ratio between the absorbance value of the sera and the cutoff value. If the EI of any area is <1, the result is negative, and if EI >1 the result is positive.

PBMC-based neutralization assay

The antibodies in the autologus serum were tested against F36PC and F36VC viruses for neutralizing activity. All sera were heat-inactivated at 56ºC for 30 minutes before use. Each serum dilution was duplicated and incubated with each virus dilution (10-50 TCID50) at 37ºC for 1 hour. Then, $1x10^5$ PHA-activated PBMC was added to each sample which was then placed in a culture incubator overnight. Cells were washed twice then resuspended in 400 μl of IL-2 medium. Six replicates of normal HIV-negative serum (NHS) in control wells were plated for each assay. On Day 4, the cultured fluid was collected for p24 antigen quantitation (Vironostka HIV-1 antigen assay, Organon Teknika). The average p24 produced in the experimental replicates was compared to the average p24 produced in NHS control wells. The percent neutralization (%NT) was calculated as the percent reduction of the control (NHS) p24 produced.

%NT = (average p24 production with NHS – average p24 production in the presence of test sample)/(average p24 production with NHS) x 100

An end point of 80% or 50% NT was used as a cutoff for the neutralization assay. When neutralizing antibody titers were determined, serial two fold dilutions of sera (starting from 1:10) were used. The endpoint neutralizing antibody titer was defined as the final serum dilution that gives 50% neutralization compared with the HIV-1 negative serum control wells.

HIV-1 infectious molecular clone containing *env* (gp120) from blood and genital fluid isolates

The proviral molecular clone pNL4-3 with envelope deleted was used as template.

Digested HIV-1 *env* (gp120) amplified DNA (F36PC and F36VC), *BstNI*/*BsaI* cut from pDrive containing *env* gene, was cloned into pNL4-3 with an envelope deleted vector (pNL4-3/gp120-F36PC and pNL4-3/gp120-F36VC) and transfected into a 293-T cell line with Polyfect transfection reagent (QIAGEN, Germany) according to the manufacturer's protocol. Viral supernatants were harvested at Days 3 and 7 after transfection and frozen at -70ºC. Supernatant from the transfection was analyzed for the presence of p24 (Vironostka HIV-1 antigen assay, Organon Teknika).

To test infectivity of HIV-1 infectious pNL4-3 clones (pNL4-3/gp120-F36PC and pNL4-3/gp120-F36VC), 500 pg of each virus in supernate was inoculated into SupT1 $1x10^5$ cells and $1x10^5$ infected pNL4-3/gp120-F36PC and pNL4-3/gp120-F36VC PBMCs were added into the Hela cell line. The culture supernatant was tested for HIV-1 p24 antigen production on, Days 7, 14 and 21 post-infections.

Statistic analysis

All statistical calculations were carried out with the SPSS/PC+ software (version 12.0; SPSS, Chicago, ILL). Results of wild type F36PC, wild type F36VC, V3 deletion F36PC and V3 deletion F36VC immune responses were compared using the Mann-Whitney *U* test.

RESULTS

Three dimensional structure of HIV-1 gp120 protein

To predict the three dimensional (3-D) structure of gp120 protein from blood (F36PC) and genital fluid (F36VC), their amino acid sequence alignments were uploaded to a predict protein program at http://cubic.bioc.columbia.edu/predictprotein. The results were viewed using the freeware program Spdby, where the molecular surface of the proteins was computed. Of gp120 406 amino acids, there were 6 discordant amino acid positions in gp120 amino acid sequences of the blood (F36PC) and genital fluid (F36VC) HIV-1 isolates at positions 26 (G^{26} and D^{26}), 104 (T^{104} and I^{104}), 140 (E^{140} and K^{140}), 282 (K^{282} and Q^{282}), 301 (K^{301} and G^{301}), and 321 (L^{321} and P^{321}). These positions may influence the conformational structure of gp120.

The 3-D structures of 2B4CG gp120 and 1RZJG gp120 proteins were used as template models for wild type (WT) and ΔV3 gp120 protein prediction from blood (F36PC) and genital fluid (F36VC) gp120 protein as shown in Figs 1 and 2, respectively. There was a β-strand at the V3 turned loop in the wild type F36VC gp120 protein, which was not found in the wild type gp120-F36PC protein.

Recombinant gp120 protein production from *E. coli* and COS-7 cells

The 51 kDa recombinant protein gp120 (rgp120F36PC/*E.coli* and rgp120F36VC/*E.coli*) produced in *E.coli* and the 110 kDa recombinant gp120 protein (rgp120F36PC/COS-7 and rgp120F36VC/COS-7) produced in the COS-7 cell line were reacted against HIV-1 seropositive serum as shown in Fig 3A and 3B, respectively. The yields of the rgp120/*E. coli* and rgp120/COS-7 were 1 mg per 100 ml culture medium and 0.3 mg per 100 ml of cells, respectively.

Because of the presence of the β-strand at the V3 turned loop of F36VC gp120 protein, recombinant V3 deleted gp120 protein was produced to compare with the wild type strain. The ΔV3 gp120 containing recombinant plasmids, pBAD/ΔV3 gp120F36PC/*E. coli* and pBAD/ΔV3gp120F36VC/*E. coli* were constructed and 0.02% arabinose was used to induce the expressed polyhistidine-tagged proteins. The yield rate of V3 deleted recombi-

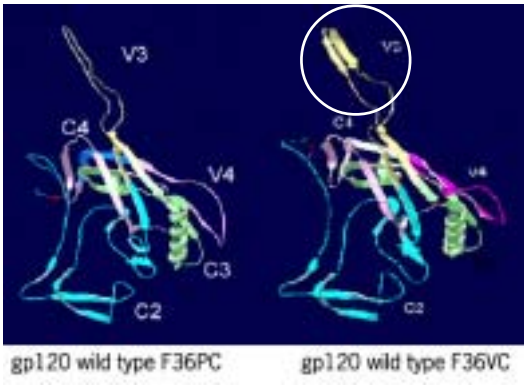

Fig 1–Three dimensional structures of gp120 proteins from wild type HIV-1 blood (F36PC) and genital fluid (F36VC) isolates.

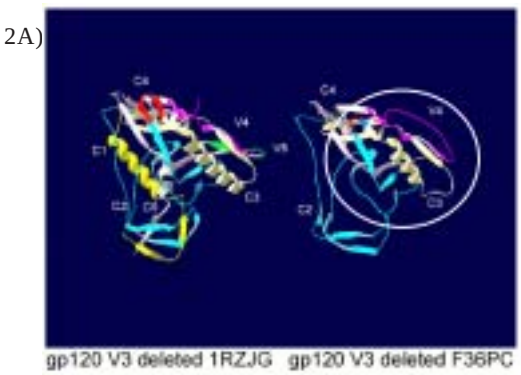

Fig 2–Three dimensional structures of ΔV3gp120 proteins from HIV-1 blood (F36PC) and genital fluid (F36VC) isolates. 2A) Ribbon diagram of gp120 from a template model (1RZJG) and ΔV3gp120 from a F36PC blood isolate. 2B)Ribbon diagram of gp120 from a template model (1RZJG) and ΔV3gp120 from a F36VC genital fluid isolate. 2C) Molecular surface of wild type (Left) and ΔV3gp120 from F36PC (Right) (V3 deleted, gp120 exposed, more accessible to C3, yellow, and V4, green, compared to the wild type).

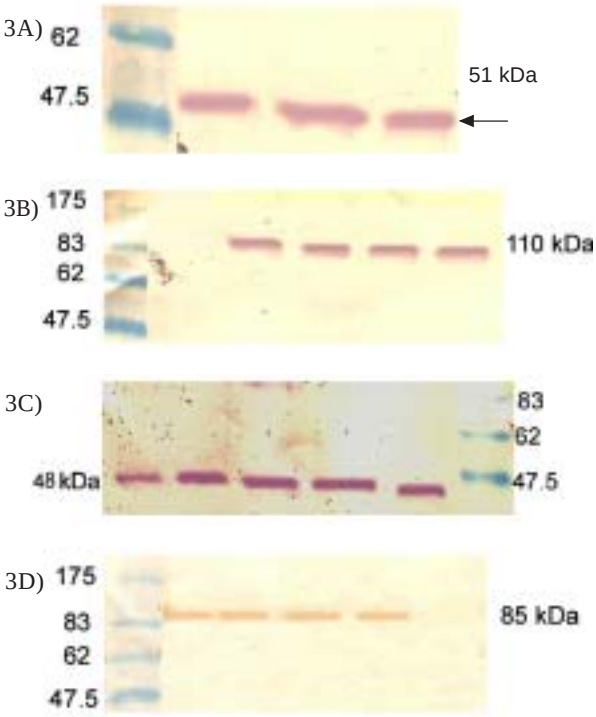

Fig 3–Western blot analysis of polyhistidine-tagged rgp120 expressed in **3A)** bacteria (rgp120-F36PC/*E. coli* and rgp120-F36VC/*E. coli*); **3B**) mammalian cells (rgp120-F36PC/COS-7 and rgp120-F36VC/COS-7); **3C)** bacteria (ΔV3rgp120-F36PC/*E. coli* and ΔV3rgp120-F36VC/*E. coli*); **3D)** mammalian cells (ΔV3rgp120-F36PC/COS-7 and ΔV3rgp120-F36VC/COS-7).

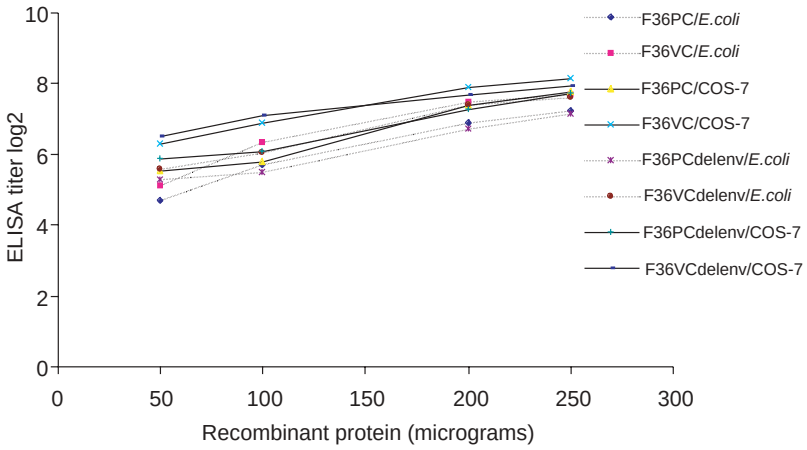

Fig 4–Dose responsive curve of antibody response, measured by ELISA, in mice immunized with recombinant gp120 proteins (F36PC/*E. coli* (—◆—) and F36VC/*E. coli* (—■—) produced in *E. coli* cells in amounts of 50, 100, 200 and 250 μg ; recombinant V3 deleted gp120 proteins in *E. coli* (V3ΔF36PC/*E.coli*-(**X**) and V3ΔF36VC/*E. coli*-(⬣) in amounts of 50, 100, 200 and 250 μg ; recombinant gp120 proteins (F36PC/COS-7 (▲) and F36VC/COS-7 (**x**) produced in mammalian cells at concentrations of 50, 100, 200 and 250 μg ; recombinant V3 deleted gp120 proteins in mammalian cells (V3ΔF36PC/COS-7(+) and V3ΔF36VC/COS-7(-) produced in amounts of 50, 100, 200 and 250 μg.

nant gp120 protein in the *E. coli* (ΔV3rgp 120F36PC/*E. coli* and ΔV3rgp120F36VC/*E. coli*) with a size of 48 kDa were 1.5 and 1.7 mg/ml, respectively (Fig 3A and 3B). Also, the stable COS-7 expressed V3 deleted gp120 protein cell lines (pSec/ΔV3gp120F36PC/COS-7 and pSec/ΔV3gp120F36VC/COS-7) were constructed. The yield rates of V3 deleted recombinant gp120 proteins in COS-7 cells (ΔV3rgp 120F36PC/COS-7 and ΔV3rgp120F36VC/COS-7) with a size of 85 kDa were 0.4 and 0.7 mg/ml, respectively (Fig 3C and 3D).

Immunogenicity in mice

To test the immunogenicity properties of recombinant protein gp120 and ΔV3rgp120 from blood and genital fluid viruses (F36PC and F36VC), mice were immunized with 50-250 μg of these recombinant proteins produced in *E. coli* and COS-7 cells. Immunogenicity was tested with an ELISA and neutralization assay and the dose responsive curve was formed by plotting the concentration of the protein against the ELISA titer as shown in Fig 4. The ELISA titers of mice sera immunized with rgp120F36PC/*E. coli* (titer 118.8 ± 24.6), rgp120F36VC/*E. coli* (titer 176.4 ± 6.6), rgp120F36PC /COS-7 (titer 168.4 ± 9.8) and F36VC-rgp120/COS-7 (titer 237.1 + 2.5) were not significantly different ($p > 0.05$).

The antibody responses from both ΔV3rgp120F36VC/*E. coli* (titer 237.1 ± 3.5) and ΔV3rgp120F36VC/COS-7 (titer 204.6 ± 3.8) proteins from genital fluid isolates were not significantly higher in immune response than ΔV3rgp120F36PC/*E. coli* (titer 119.3 ± 39.6) and ΔV3rgp120F36PC/COS-7 (titer 152.5 ± 5.5) produced from blood isolates ($p > 0.05$). Antibody responses from wild type and ΔV3 rgp120 proteins were not significantly different ($p > 0.05$) (Fig 4).

Neutralizing antibody levels from rgp120F36VC/COS-7 (titer 90 ± 15.6) and rgp120F36PC/COS-7 (titer 36 ± 10.8) against homologous virus were found, but neither was significantly higher than the corresponding recombinant proteins at 250 μg as shown in Table 1. Comparing neutralizing antibody levels between rgp120F36VC/COS-7 and rgp120F36PC/COS-7, we found no statistically significant differences ($p>0.05$). The antibody titers from the wild type rgp120F36

Table 1
Neutralizing antibody titers (NT titers) of sera from mice immunized against F36PC and F36VC viruses. Sera from 3 mice per recombinant protein immunization were collected 1 week after last immunization with 250 μg of recombinant protein.

Recombinant proteins	Neutralization Titer				ELISA titer	
	F36PC isolate	*p*-value	F36VC isolate	*p*-value	GMT (±SD)	*p*-value
rgp120F36PC/*E.coli*	<10		<10		118.8 ± 24.6	
rgp120F36VC/*E.coli*	<10	0.07	<10	0.07	176.4 ± 6.6	0.55
rgp120F36PC/COS-7	36 ± 10.8		<10		168.4 ± 9.8	
rgp120F36VC/COS-7	<10	0.08	90 ±15.6	0.06	237.1 ± 2.5	0.06
pBAD/ΔV3 gp120F36PC/*E.coli*	<10		<10		152.5 ± 5.5	0.065
pBAD/ΔV3 gp120F36VC/*E.coli*	<10	0.07	<10	0.07	119.3 ± 39.6	
pSec/ΔV3gp120F36PC/COS-7	<10		<10		237.1 ± 3.5	0.06
pSec/ΔV3gp120F36VC/COS-7	<10	0.07	<10	0.07	204.6 ± 3.8	

Table 2
The average p24 Ag level of infectious viruses pNL4-3/gp120-F36PC and PNL4-3/gp120-F36VC detected after transfection in 293-T cells, SupT1 cells and Hela cell.

Cell line	P24 Ag (picograms)					
	pNL4-3/gp120-F36PC			pNL4-3/gp120-F36VC		
	Day 3	Day 7	Day 14	Day 3	Day 7	Day 14
293-T cell	150.05	149.50	ND	159.05	148.50	ND
SupT1	12.5	11.5	100.40	13.5	12.80	120.25
Hela cell	9.7	10.5	50.4	8.6	11.6	102.6

ND = not done

VC/COS-7 (titer 90 ± 15.6) and the deletion of V3 pSec/ΔV3gp120F36VC/COS-7 (titer < 10) were not significantly different ($p > 0.05$).

HIV-1 infectivity assay

The HIV-1 molecular clone containing *env* (gp120) gene from F36PC and F36VC isolates were constructed by replacing the *env* (gp120) gene of the pNL4-3 with the *env* (gp120) gene from the F36PC or F36VC isolates (pNL4-3/gp120-F36PC and pNL4-3/gp120-F36VC). The p24 antigen in these two infectious clones was detected on Day 3 after transfection in the amounts of 150.25 pg for pNL4-3/gp120-F36PC and 159.05 pg for pNL4-3/gp120-F36VC. The amount of virus used in the infectivity assay was chosen by varying the p24 antigen concentration: 250 pg, 500 pg and 1,000 pg to infect the SupT1 cell. By Day 14 the infectious virus had average p24 antigen production levels of 100.4 pg and 120.25 pg for pNL4-3/gp120-F36PC and pNL4-3/gp120-F36VC, respectively. The p24 antigen production in the pNL4-3 SupT cell line on Day 14

was 220 pg (Table 2). On Day 14, the amount of virus in the Hela cells from the genital isolates (pNL4-3/gp120-F36VC, 102.6 pg) was higher than from the blood isolates (pNL4-3/gp120-F36PC, 50.4 pg).

DISCUSSION

Safety considerations surrounding other more conventional strategies have led some vaccine makers to pursue more modern approaches when designing an AIDS vaccine. Theoretically, virus-specific proteins, or portions of the proteins (peptides), could be used to induce virus specific immunity without any possibility of inducing HIV disease. Protein subunit vaccines are made by genetically engineering HIV genes of interest in a laboratory culture system, such as bacteria, yeast, insect, or mammalian cell cultures, then purifying the resulting proteins from the culture media. Peptide vaccines are made by chemically synthesizing the region of interest. Generally, those regions include known T-cell or B-cell epitopes. Envelope subunit and peptide approaches were amongst the earliest attempts to make an HIV vaccine, based on the premise that the envelope protein would be the most important target because neutralizing antibodies in HIV infected persons target the envelope, are a major viral surface glycoprotein and a key mediator of the entry process (Earl *et al*, 1991; Doms *et al*, 1993; Saikai *et al*, 1993; Yu *et al*, 1993; Chan *et al*, 1997; Weissenhorn *et al*, 1997; Young, 1999).

Thus, virus-specific envelope proteins have been the primary target of these vaccine strategies with the goal of producing an immunogen that induces virus neutralizing antibodies. However, protein and peptide subunit strategies are also being pursued as a way of inducing T-cell immunity targeting both envelope and *gag*-gene specified virus structural proteins. Envelope protein vaccines, including insect, yeast, and mammalian cell derived products, were some of the earliest preparations tested in human trials, with the full-length *env* gene product (gp160) and the surface glycoprotein (gp120) tested in phase I trials. The mammalian cell-derived gp120 product was later tested in phase II trials, and large-scale phase III trials are currently being carried out on two of these products. Vaccines currently being tested in phase III trials in Thailand include envelope proteins derived from CCR5-using primary isolates and are produced by VaxGen. The results showed the vaccine neither prevented infection nor slowed disease progression in those who became infected after receiving the vaccine (McCarthy, 2003).

Attempts to boost specific immunity with HIV-1 recombinant glycoprotein (RGP) vaccines have resulted in some HIV infected individuals developing augmented humoral and cellular immune responses (Modrow *et al*, 1987; Leonard *et al*, 1990; Helseth *et al*, 1991). Whether vaccine associated immune responses correlate with any changes in the repertoire of the viral quasispecies remains unknown. Kwong *et al* (1998) suggested that RGP vaccination in these children did not drive viral evolution. Indeed, there may have been an inverse relationship between vaccine-associated immune response and the degree of viral diversity.

Many investigators believe that virus envelope immunogens need to be structurally folded to mimic their appearance in infectious virus particles to induce functional neutralizing antibodies. To pursue this theory, attempts are being made to generate envelope proteins that have been stabilized in their wild-type trimeric configuration. Recent molecular studies indicate the envelope proteins undergo structural changes after binding to cellular receptors; this information has led some investigators to design immunogens that mimic these structural changes. In addi-

tion, some investigators are making envelope mutants, increasing or decreasing the number of glycosylation sites or removing portions of the coding sequence, in an effort to increase the immunogenicity of the proteins. Although none of these envelope protein studies have progressed to phase I testing, the results of animal studies have been promising.

Ninety percent of HIV-1 infections worldwide are transmitted heterosexually. The genital mucosa is the site of initial contact with HIV-1 for most exposed individuals. HIV-1 infection is characterized by tremendous genetic variation. Compartmentalization, the occurrence of distinct yet phylogenetically related HIV-1 genotypes in different anatomic sites, has been well documented in treated and untreated individuals alike. Compartmentalization occurs in diverse tissues, including plasma, brain, and lung tissue (Beretta and Dalgleish, 1994; Parren *et al*, 1997; Wyatt *et al*, 1998). Studies of both women and men have revealed viral variants in the genital tract that differ from those in the blood (Wu *et al*, 1996; Farzan *et al*, 1998). Six discordant amino acids in the gp120 amino acid in blood (F36PC) and genital secretion (F36VC) isolates were found. The difference in amino acids in the HIV-1 envelope protein from the blood and genital secretions may influence the structure of the envelope protein. Thus, F36PC and F36VC isolates were used as templates to produce recombinant envelope (gp120) protein in *E. coli* and mammalian cells. The production of wild type rgp120F36PC and rgp120F36VC proteins in *E. coli* (1.0 mg/ml) was higher than in mammalian cells (0.3 mg/ml). One reason to explain this is codon bias. In general, the more codons a gene contains that are rarely used in expression, the less likely it is the heterologous protein will be expressed at reasonable levels. Low expression levels happen when rare codons appear in clusters or in the N-terminal part of the protein (Kwong *et al*, 2000).

The antibody response against wild type rgp120F36VC protein from genital secretion isolates was higher than against rgp120-F36PC, but the difference was not statistically significant ($p > 0.05$). The three dimensional structure of rgp120F36VC contained extra β-strands at the V3 loop but there were no differences in immunogenicity or infectivity in lymphocyte cells when compared with rgp120F36PC recombinant protein. However, the infectivity assay in epithelial cell line of pNL4-3/gp120F36VC was better than with the cell line pNL4-3/gp120F36PC. The extra β-strands in the V3 loop may be involved in cell tropism. Recombinant protein is not a particularly effective immunogen, and a number of approaches have been suggested to enhance its immunogenicity. The goal is to provide an antigen that induces the generation of antibodies with the ability to neutralize primary isolates of HIV-1 from diverse clades. A variety of studies have shown that removal of the hypervariable loops of recombinant HIV-1 gp120 creates a molecule that retains its structural integrity, while providing enhanced accessibility to specific domains of the conserved regions of this glycoprotein, notably the CD4 binding domain (CD4bd) and C1 regions (Brown *et al*, 1994; Choe *et al*, 1996). The structure change results in a change in antibody response, by deletions in the major variable region, V3, from gpl20F36VC and gp120F36PC. An overlapping extension method was performed to delete V3 from gp120 in this study.

Production of wild type rgp120F36PC and F36VC proteins in *E. coli* (1.0 mg/ml and 1.2 mg/ml) and mammalian cells (0.3 mg/ml and 0.5 mg/ml) are lower than ΔV3rgp120 F36PC and ΔV3F36VC in *E. coli* (1.5 mg/ml and 1.7 mg/ml) and mammalian cells (0.4 mg/ml and 0.7 mg/ml), respectively. These results

are consistent with a study by Wyatt *et al* (1993), who found approximately four to fivefold more processed ΔV3 glycoprotein appeared in the supernatant than was observed with the wild-type glycoprotein. Deletion of V3 exhibited slightly less of an effect on antibody response. This is consistent with a study by Kim *et al* (2003), who found the same level of antibody response with the wild type recombinant gp120, ΔV1-2, ΔV3 ,ΔV4 and ΔV1-3 but is in contrast to a study by Lu *et al* (1998) who compared the immunogenicity of three different wild types and a V1/V2/V3 deleted envelope. The results show the variable loop deleted antigens were better than the wild type in eliciting antibodies (Dumonceaux *et al*, 1998). The predicted three dimensional structures of rgp120F36PC and rgp120F36VC were shown at two different points in the subregion of V3. 1) The nearby tip of the V3 region of F36VC exhibited antiparallel β-strands while helic was found in the V3 region of F36PC. Different structures may affect the antibody response of rgp120F36VC, which was higher than that of rgp120F36PC. The functions of the different structures are still unknowns. The deletion of V3 did not enhance the structure change. Partial or subregion deletion of V3 may be the best approach to effect structural change and antibody response. 2) The structure of wild type rgp120F36PC before V3 deletion exhibits a coil at the C3 region. After V3 deletion, the coil in the C3 region is decreased; only 3 coils were found on gp120F36PC/ΔV3. When compared to the molecular surface of wild type F36PC, in F36PC/ΔV3 C2, C3 and V4 were more accessible after V3 deletion (data not shown). The latter structural change did not affect the antibody response of rgp120 F36PC.

Efforts to enhance the immunogenicity of HIV-1 envelopes with respect to the capacity to induce primary virus-neutralization antibodies have included removal of variable loop sequences and mutation of predicted N-linked glycosylation sites, based on the hypothesis that these structures may cloak important neutralization epitopes (Bandres *et al*, 1998; Dumonceaux *et al*, 1998). Our study showed that the V3 deleted recombinant envelope proteins (ΔV3rgp120F36PC and ΔV3rgp120F36VC) did not exhibit neutralization antibody responses, which is consistent with the finding of Kim *et al* (2003). The variable loop deletion mutant envelope constructs elicited neither more potent nor broadly cross reactive neutralizing antibodies to the wild type envelope (Kim *et al*, 2003). The use of envelope constructs with less drastic deletions of the variable loops may result in a better outcome (Dumonceaux *et al*, 1998).

To confirm the viruses gained replication capacity, F36PC and F36VC viruses were directly compared in an infection experiment. An HIV-1 infectious molecular clone was produced. The results show the viruses which contained F36PC and F36VC envelopes were replicated efficiently.

We found that HIV-1 envelope proteins from genital fluid isolates elicited higher antibody responses than envelope proteins constructed from blood isolates but the difference was not statistically significant. The deletion of V3 from the envelope will not enhance the neutralization antibody response when compared to the wild type. The deletion of the subregion V3, at the N-linked glycosylation site and optimization of V1/V2 may enhance the antibody response.

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