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**IMPACT OF STRUCTURAL CONFORMATION OF THE V3 LOOP ON THE
IMMUNOGENIC PROPERTIES OF gp120 ENCODED IN HIV-1 FIELD ISOLATES**

by

Sang-Kyung Lee

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

2000

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Abstract**IMPACT OF STRUCTURAL CONFORMATION OF THE V3 LOOP ON THE IMMUNOGENIC PROPERTIES OF gp120 ENCODED IN HIV-1 FIELD ISOLATES.****By****Sang-Kyung Lee**

Adviser: Dr. William Boto

The results of the study presented in this thesis show that clones of the ENV gene derived from genetically divergent HIV-1 field isolates fall into two major subsets based on the predicted secondary structure of the V3 region in gp120. One subset exemplified by the clones A-UG06c, B-RT3.12 and C-UG045 is predicted to assume a β -turn conformation in the V3 loop, and comprises the apical tetrapeptide GPGX. The other subset exemplified by the clones D-UG23c and D-UG042 (apical tetrapeptide GXGX) are deficient in the expression of the β -turn in the loop. Since secondary conformations are highly likely to confer antigenic properties in a protein backbone at least for B cells, I have used nucleic acid immunization to test the effect of the β -turn deficiency on the immunogenic potential of rgp120 encoded in these field isolates. As hypothesized, inoculation of BALB/c mice with the ENV plasmid encoding the β -turn-expressing rgp120 molecules resulted in the development of a vigorous antibody response to the homologous V3 loop peptides. In contrast, immunization with an rgp120 clone which is deficient in the β -turn in the V3 loop showed no evidence of antibody development to the loop. Instead, the latter clones triggered T cell proliferative responses and markedly increased levels of IL-2 and IFN- γ production by T cells. Significantly, reconstitution of

the β -turn conformation by site-directed mutagenesis of the V3 loop yielded rgp120 molecules which restored antibody production while diminishing the cell-mediated immune (CMI) responses to the loop. These observations demonstrate a marked impact of genetic variation on secondary conformation and immunogenic properties of gp120 encoded in divergent HIV-1 field isolates.

To

My wife, Soo-Ok, for her continual support and encouragement

My son, Jung-Soo and my daughter, Sang-Ah, for being there

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INTRODUCTION

Several features of HIV-1 property and infection must be considered for the preparation of a vaccine (1-3) that induces strong cellular and humoral immune responses (4). Toward this objective, it is important to identify which antigens to use. In this regard, vaccination studies with envelope protein from field isolates are necessary to search for proper immunogens.

The human immunodeficiency virus type 1 (HIV-1) is a lentivirus associated with the cause of acquired immune deficiency syndrome (AIDS). The clinical syndrome characterized by a marked reduction in the number of CD4⁺ cells and the development of opportunistic infections and cancer, results from the persistent replication and spread of HIV-1 in the body. The transmission of HIV-1 is mediated by the glycoprotein gp120 (Fig. 1), located on the virion surface: gp120 contains the binding site for CD4 receptor and it is the major neutralizing immunogen. The envelope glycoproteins gp120/41 are processed from a precursor molecule, gp160, which is encoded by the ENV gene and a singly spliced transcript from the full-length viral mRNA (Fig. 2) (5, 6). The HIV-1 genome encodes 2 structural proteins (gag and env), 3 enzymes (reverse transcriptase (RT), integrase, protease) and 6 viral regulatory proteins tat, vif, vpr, vpx, nef, and rev (7).

Infection begins with the attachment of gp120 to its receptor, CD4, which is expressed on the cell surface (8, 9) and conformational changes in both the viral envelope and CD4 receptor permit the binding of gp120 to other cell surface co-receptor molecules, notably the chemokine receptors CXCR-4 and CCR5 (Fig. 3) (10-12). This

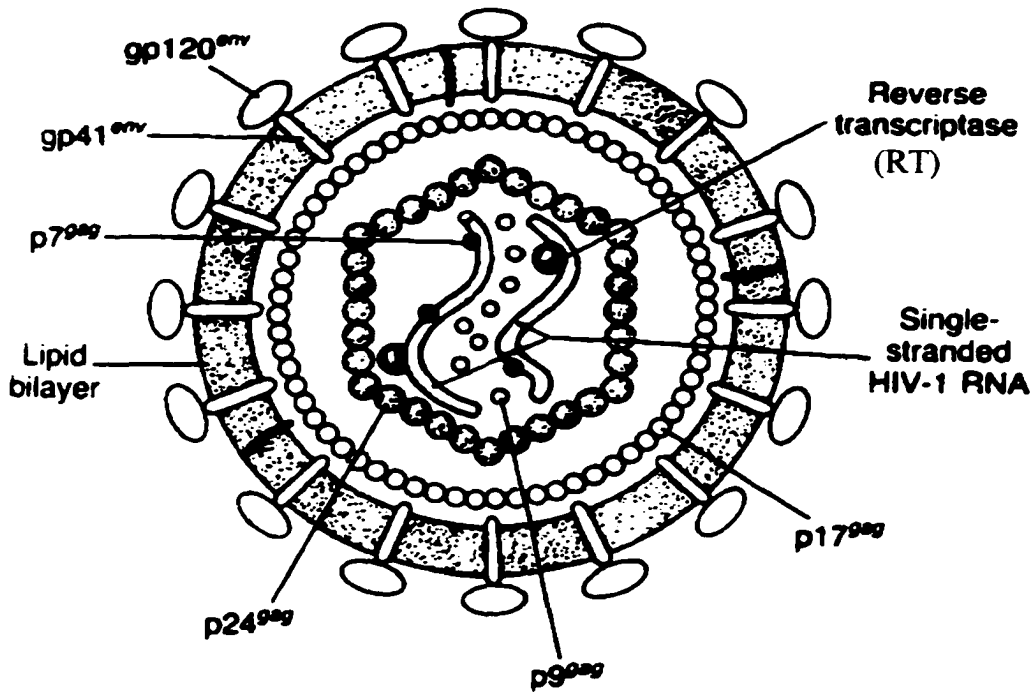


Figure 1. HIV-1 virion: structural and regulatory proteins. Structural proteins (gp120, gp41, p24, p17, P9, p7) and regulatory protein (reverse transcriptase [RT]) are shown (adapted from reference 5).

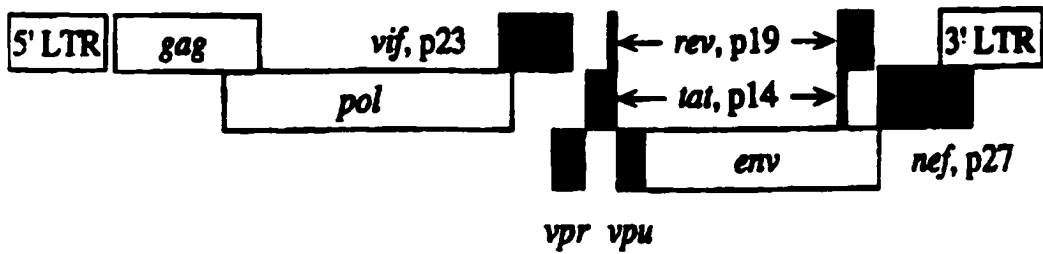


Figure 2. Genetic map of HIV-1 showing structural and regulatory genes. Vpu is encoded in HIV-1 and Vpx is formed HIV-2.

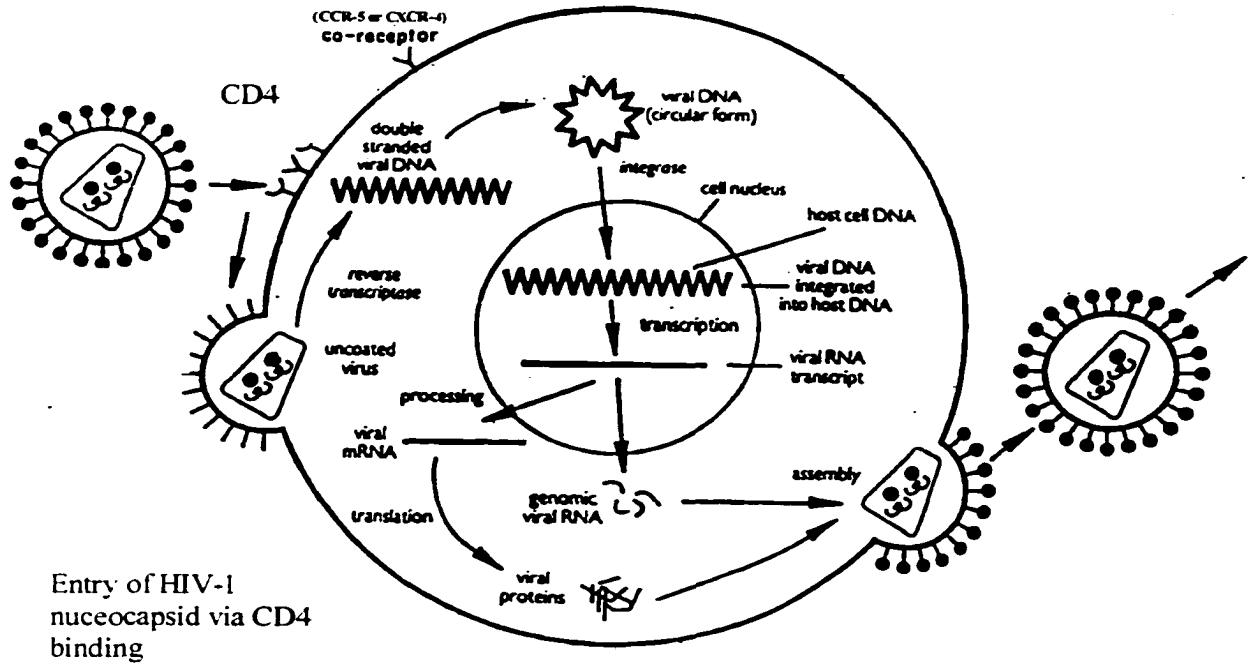


Figure 3. Replication cycle of HIV-1. Binding to receptor CD4 and co-receptor (CXCR-4 or CCR-5) initiates viral entry. (adapted from reference 13).

second attachment brings the viral envelope in contact with the cell surface, allowing interaction between gp41 on the viral envelope and the cell surface. Subsequently, the viral nucleocapsid enters the cell. Once the virus/genome has entered the cell, the RNA undergoes reverse transcription and eventually forms a double-strand DNA copy. This cDNA is transported to the nucleus where it integrates into the cellular chromosome. The integration of the HIV-1 provirus appears to be random and is essential for the cells to produce progeny virions. Following virus integration, activation of the host is also required for the initiation of transcription of the provirus into either viral genomic or messenger RNA (mRNA). HIV-1 mRNA is subsequently translated into functional proteins. Virion assembly takes place in the cytoplasm, where genomic RNA is incorporated into the capsid and the mature virions bud from the plasma membrane (Fig. 3) (13). Further, transmission of HIV-1 requires the successful interaction of the virus with the co-receptors (CXCR-4 or CCR-5) on the cell surface (fig. 3). The natural ligand for CXCR-4 is the chemoattractant stromal-cell-derived factor 1 (SDF-1) and this cytokine can block syncytium-inducing (SI) HIV-1 infection of T-cell lines (7, 14, 15). SDF-1 is a member of a family of low-molecular-weight cytokines called chemokines. The other ligand for macrophage-tropic non-syncytium inducing (NSI) strain of HIV-1 is the receptor for β -chemokines RANTES, macrophage inflammatory protein 1 α (MIP-1 α), and MIP-1 β . The receptors for β -chemokines are CCR-5, CCR-3 and CCR-2b (11, 12, 15, 16). These receptors are not used by the T cell tropic SI strains. Interestingly, the transition from non-syncytium inducing to syncytium-inducing (NSI \rightarrow SI) viral phenotypes is also accompanied by switching in co-receptor usage (Fig. 4) (15, 17).

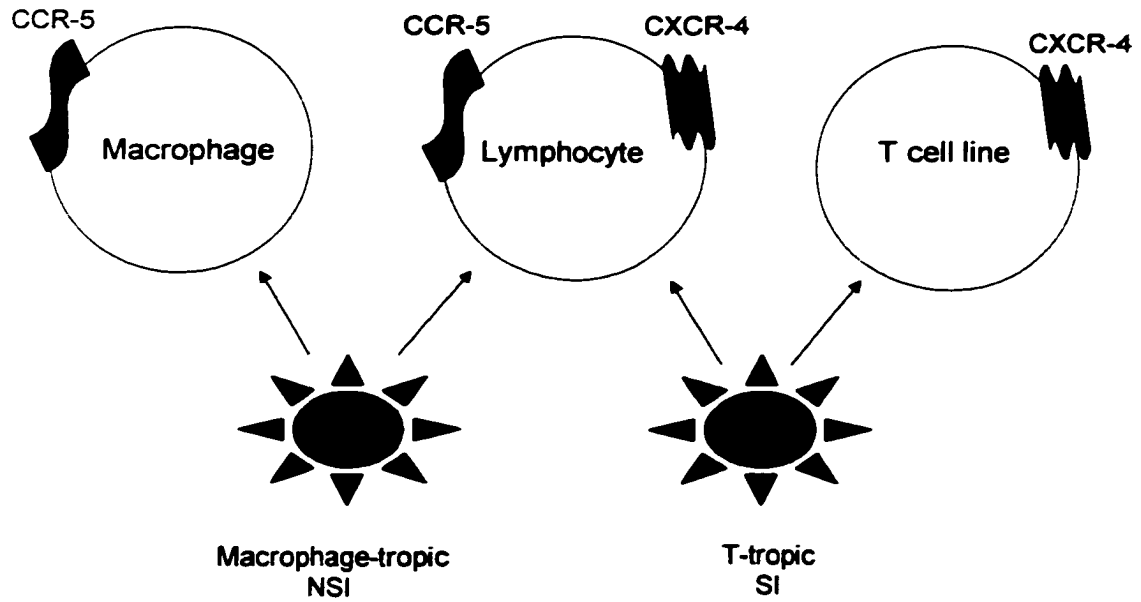


Figure 4. Model for HIV-1 co-receptor usage. Transmission of initial infective HIV-1 is mediated by macrophage tropic NSI virus. In the later stage of infection the virus undergoes the amino acid mutation in V3 loop and broadens the usage of co-receptor, to include CXCR-4 and the initial NSI phenotype becomes a T cell tropic SI virus.

Recently, it has been suggested that dendritic cells (DC) promote efficient infection in-trans of cells that express CD4 and chemokine receptors. The DC-specific C-type lectin, DC-SIGN, efficiently captures HIV-1 in the periphery and migrates to secondary lymphoid organs rich in T cells. Upon arrival, DC will cluster with T cells and DC-SIGN enhances HIV-1 infection of T cells in-trans, leading to a productive and sustained infection (Fig. 5) (18).

Major obstacles to the development of a globally efficacious vaccine for HIV/AIDS persist at several levels. One of the drawbacks is the impact of MHC polymorphism on the vigor of immune response elicited by HIV-1 antigens. The concern for MHC polymorphism is based on studies performed in animals and humans. Berkower and Bridgewater (19) have shown that H-2 congenic strains of mice exhibit a marked difference in the magnitude of immune response induced by the leading candidate vaccine gp120. This finding is consistent with a report indicating that homozygosity for certain alleles at the HLA-A or -B locus is a strong predisposing factor for rapid progression to AIDS in HIV-1 seropositive African and Caucasian populations (20). HIV-1 is a relatively new virus which has rapidly evolved into an infectious and highly fatal agent within the last 35 years. The apparent disparity in the quality of immune response to gp120 would suggest that the HLA class I/II repertoire has not sufficiently evolved to engage all major variants of the antigen.

The other area of concern is the impact of genetic and antigenic variation of HIV-1 on vaccine efficacy. The accumulation of mutations in the virus is particularly pronounced for the ENV, a region of the genome which encodes gp120/41 (Fig. 6).

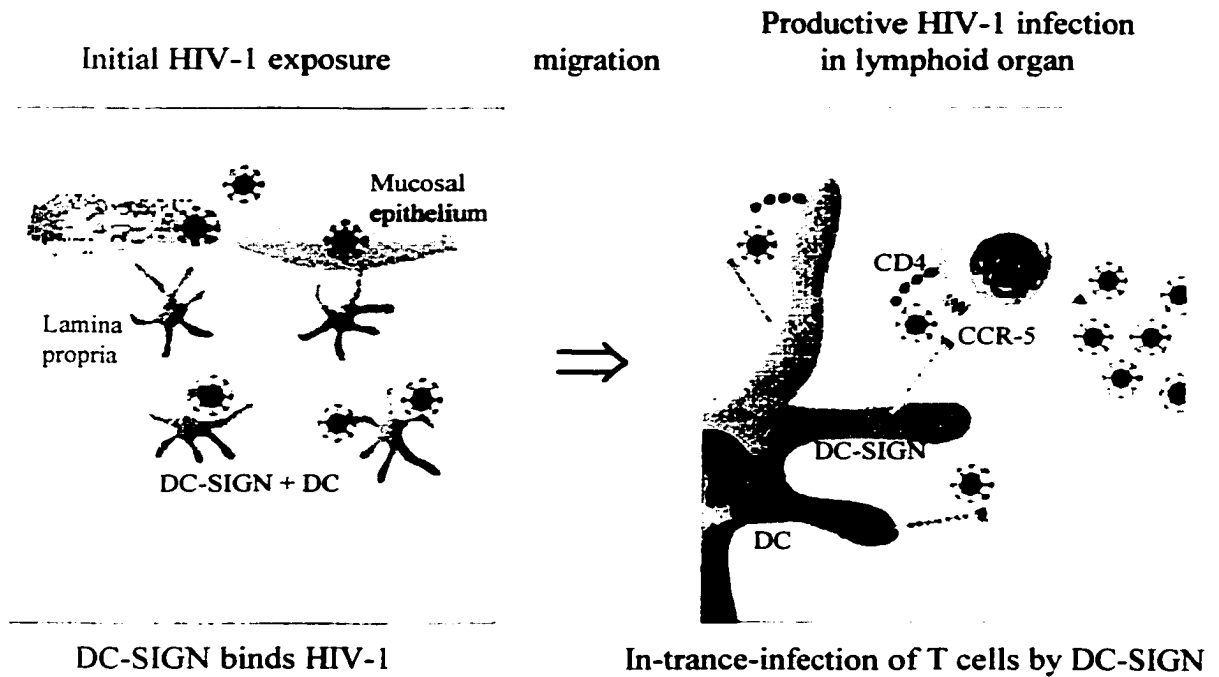


Figure 5. Model of HIV-1 assumes that DC-SIGN serves as a trans-receptor after initial exposure. DC-SIGN captures HIV-1 and retains long-term infectivity of the virus. DC are the primary cells targeted by HIV-1 during mucosal exposure, and are DC-SIGN positive. (adapted from reference 18).

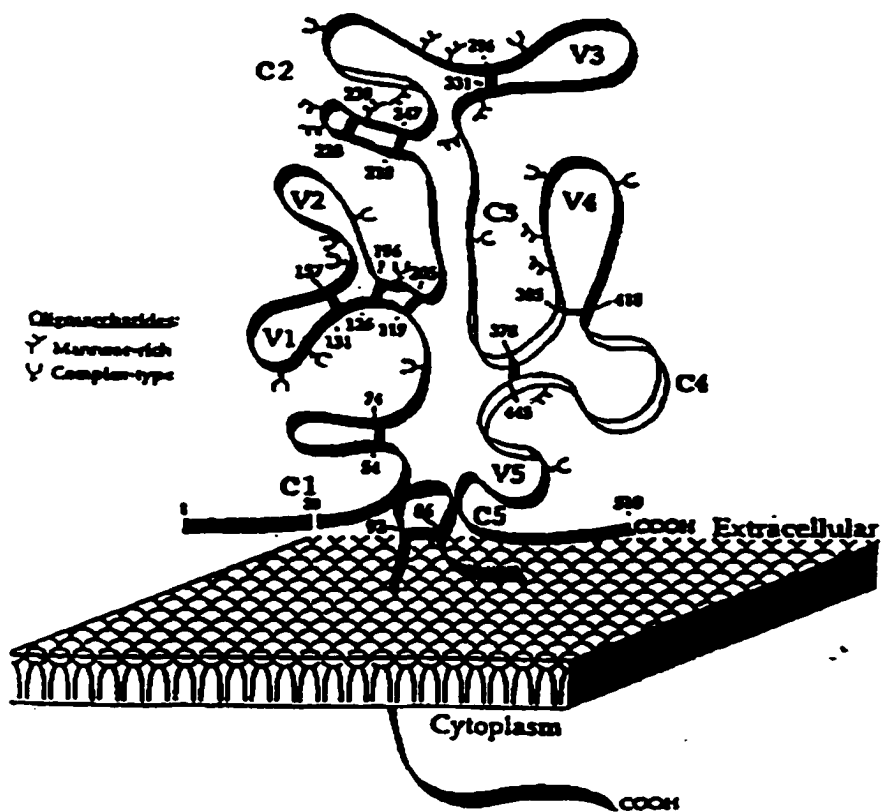


Figure 6. Schematic representation of the secondary conformation of gp120 (HIV-1IIIB). The variable regions (V1 to V5) and the conserved regions (C1 to C5) are shown (adapted from reference 21).

To date three major groups of HIV-1 have been identified based on phylogenetic analysis of ENV or GAG (21, 22). The results of these studies show that most of the infections are caused by the M group which comprises the virus subtypes A through K (21-23). The remainder of the infections have been attributed to the outgroup O (24) and the recently described group N (25, 26) viruses. The N and O subtypes appear to be confined to a region in West Africa (24-26). In one instance, co-infection with the M and O subtypes in this region of Africa has been reported to yield a recombinant virus with unique genomic sequence and replicative capacity (27). The molecular evolution of the existing strains and the emergence of new recombinant viruses are likely to further complicate vaccine development. A systematic study of the impact of amino acid variation on conformational structure which may affect the antigenic properties of HIV-1 immunogens is fundamental to vaccine research and development efforts.

Based on studies of HIV-1 infected individuals over time, cytokines produced by TH1 (IL-2, IFN- γ) and TH2 (IL-4, IL-10) cells play a significant immune regulatory role in HIV-1 infection and affect progression to disease (28, 29). TH1 cells produce IFN- γ and IL-2 and promote cell-mediated effector responses; whereas TH2 cells produce IL-4, IL-5, IL-6 and IL-10 cytokines which influence B cell reactivity and elicit antibody responses. The findings that T cell proliferation and IL-2 production declines while B cell response increase, suggests that a TH1 \rightarrow TH2 switch occurs in the progression of infection to AIDS (29, 30). These results indicated that TH1 responses are found primary in healthy asymptomatic individuals, whereas a predominance of the TH2 subset response occurs during the symptomatic stage of the infection (Fig. 7) (28-30).

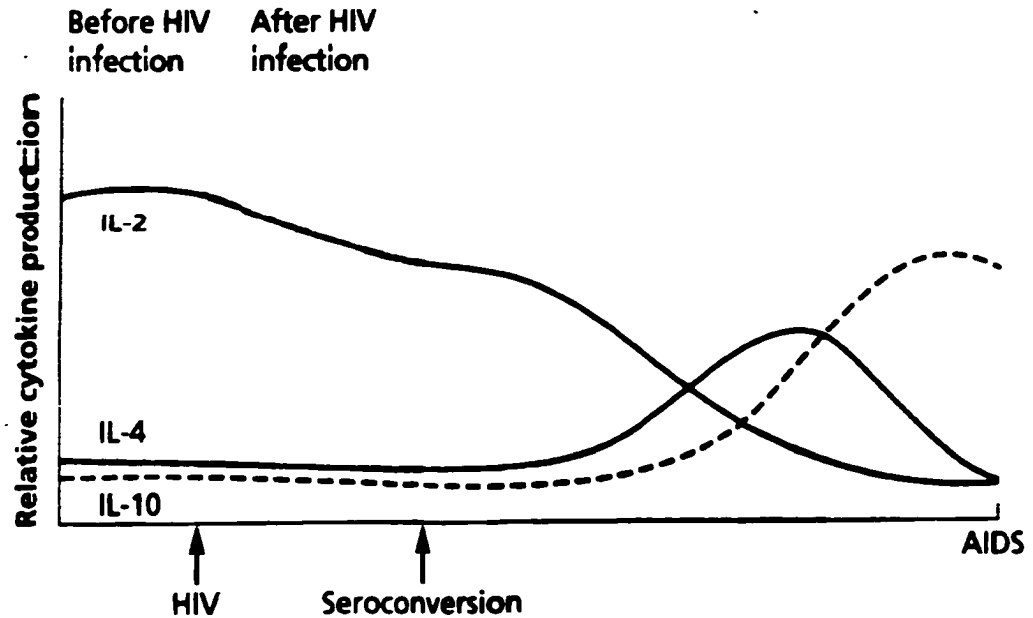


Figure 7. CD4 cytokine production before and after HIV-1 infection. Prior to infection, the TH cell produce IL-2 and IFN- γ cytokines. But with progression to disease, the TH1 response is replaced by production of the TH2 cytokines, IL-4 and IL-10 (adapted from reference 31).

Using serological analyses, our laboratory (32) and other groups (33, 34) have previously reported that gp120 derived from field and laboratory adapted HIV-1 isolates can potentially display a large number of B and T cell epitopes. Many of these determinants were shown to map to regions of high sequence diversity (32, 35, 36). The levels of amino acid variation recorded for these determinants suggest that HIV-1 is vulnerable to immunological attack and neutralization at these sites. Indeed the virus is known to express at least two major antibody neutralizing epitopes in gp120. One of the determinants is a relatively conserved conformational epitope which comprises the CD4-binding site (37-40). The other important epitope is a highly variable region which maps to the V3 loop. The V3 region has been extensively investigated and shown to include neutralizing amino acid residues located at the apex of the loop (40-42). Neutralizing serum antibodies from naturally infected donors apparently recognize the same or a closely associated structure within the loop (42-44). Further, certain neutralizing antibodies that display relatively broad reactivity against diverse isolates are reported to recognize a GPGR tetrapeptide located at the cap of the loop (41). More recent data has shown that the apical sequence GPGR is expressed in a large number of field and laboratory adapted isolates analyzed to date (21).

Studies previously performed in our laboratory have permitted the molecular cloning, structural analysis and serological characterization of gp120 derived from HIV-1 isolates of diverse subtypes and geographical origins. In the present investigation, a nucleic acid immunization technique was utilized in investigating the impact of genetic variation on the immunogenic potential of rgp120 encoded in variant field isolates. The evidence presented herein indicates that divergence in the levels of antibody or cell-

mediated immune (CMI) responses elicited by several rgp120 molecules may be significantly attributable to variations in the conformational structure of the immunogen. I demonstrate here that site-directed amino acid substitutions in the V3 loop can greatly alter immune responses to gp120 encoded in certain field isolates.

MATERIALS AND METHODS

Selection of ENV clones for immunization. We have previously described the genetic variation in ENV and the alignment of the nucleotide sequences and phylogenetic tree has been reported based on the divergence in ENV sequences (Fig. 8) (35). Among these divergent HIV-1 clones, the following isolates were used for analysis: A-UG06c, B-RT3.12, C-UG045, D-UG23c and D-UG42. The primary interest was to study the impact of this extensive mutation on primary and conformational structure which may ultimately affect the immunogenic properties of gp120 molecules encoded in these divergent field isolates.

PCR amplification of ENV from HIV-1. The ENV clones A-UG06c, B-RT3.12, C-UG045, D-UG042 and D-UG23c (Fig. 8) were recovered by polymerase chain reaction (PCR) conducted on the inserts that were previously subcloned into the pT7 PCR vectors (35, 45, 46). The ENV amplified inserts (625 bp or and 725 bp) encode the residues extending from the C2 to the V5 region of gp120 (41, 45, 47). The primers used for the amplification reactions are shown (Table 1). The primer pair P045A and P045B was used to amplify the clones B-RT3.12, C-UG045, D-UG042 and primer P23cA and P23cB were used for amplification of D-UG23c. The oligonucleotides were designed to include the Bam HI enzyme restriction site at the 5' end of the upstream (sense) and downstream (antisense) primers (Table 1). A stop codon was inserted at 5' of the Bam HI site in the downstream primer.

The PCR reaction mix contained 30 pmol of each primer, 0.5 μ g of template DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.2 mM of each dNTP

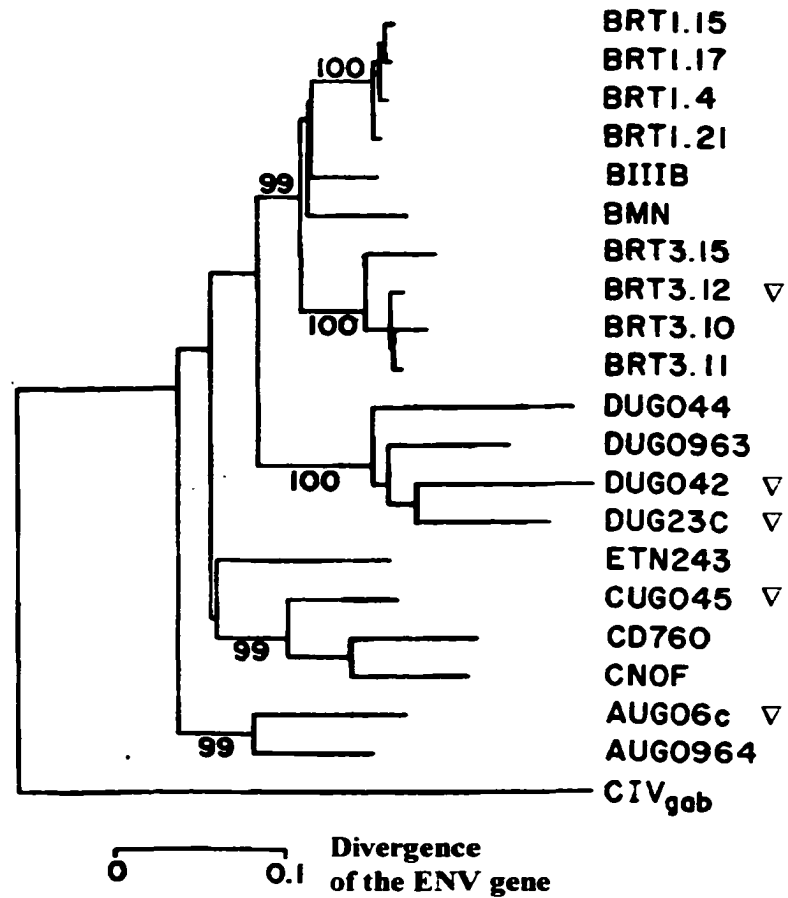


Figure 8. Phylogenetic tree of ENV showing the subtype classification of the Ugandan and New York clones. The first letter in the name of each clone indicates its genotype in accordance with Los Alamos National Laboratory nomenclature. Inverted arrow (▽) indicates ENV clones used here for the analysis of immunogenic property (adapted from reference 35).

Table 1. Primers used for the amplification of ENV clones for DNA immunization. The primers were designed to include the Bam HI (underlined) and transcription stop codon (**bold**).

Primer	Sequence	Location (BH10)
Upstream (sense)		
P23cA	5'-CCT <u>GGATCC</u> CCTCACAAACAATGCTA	5890-6915
P045A	5'-TT <u>GGATCC</u> AGTCTAGCAGAAGAAGAG	6857-6878
Downstream (antisense)		
P23cB	5'-CA <u>AGGATCCT</u> CAGAACATAGCTCCTAGTC	6711-7640
P045B	5'-TTC <u>GGATCC</u> CTAATTGTCCTCATATCTC	7495-7517

(dATP, dTTP, dGTP and dCTP, Pharmacia, NJ) and 1.5 units of AmpliTaq DNA polymerase (Perkin Elmer Cetus, CA) in a total volume of 50 μ l. Fifty microliters of mineral oil was overlaid on the PCR reaction mix to prevent evaporation. The reactions were conducted under the following conditions: denature (95 °C) 30 sec; anneal (50 °C) 30 sec; extend (72 °C) 1 min, for 35 cycles. The reaction was then subjected to a final extension at 72 °C for 10 min. The samples were cooled to 4 °C until used. The PCR products were extracted with phenol:chloroform (1:1) and precipitated with ethanol and 1/10 volume of 3 M ammonium sulfate (pH 5.2) as described (48). The pellet was dissolved in 20 μ l of TE (pH 8.0).

Construction of expression plasmids. The plasmid, CMVint-BL, was initially constructed by Chapman et al. (49) and subsequently modified to include several additional cloning sites (50). Both intron A and the human tissue plasminogen activator (TPA) signal in CMVint-BL have been shown to contribute to the increased secretion of several glycoproteins, including rgp120 (49). In the experiments conducted herein, each clone of ENV was inserted at the Bam HI site downstream of the TPA signal peptide (Fig. 9) (32).

DNA purification and sequencing. Plasmid DNA was prepared from overnight cultures of bacterial colonies in LB medium by the alkaline lysis method and precipitated with PEG-6000 (48). Purified DNA was dissolved in distilled H₂O and dried. The DNA samples were screened by restriction analysis with Bam HI to determine the appropriate orientation of the ENV insert. Selected clones were sequenced using automated DNA cycle sequencing (ABI Sequencer Model 373A and the Prism kit, Applied Biosystems, CA) with fluorescent dye-deoxy nucleotide terminators and Taq DNA polymerase. The

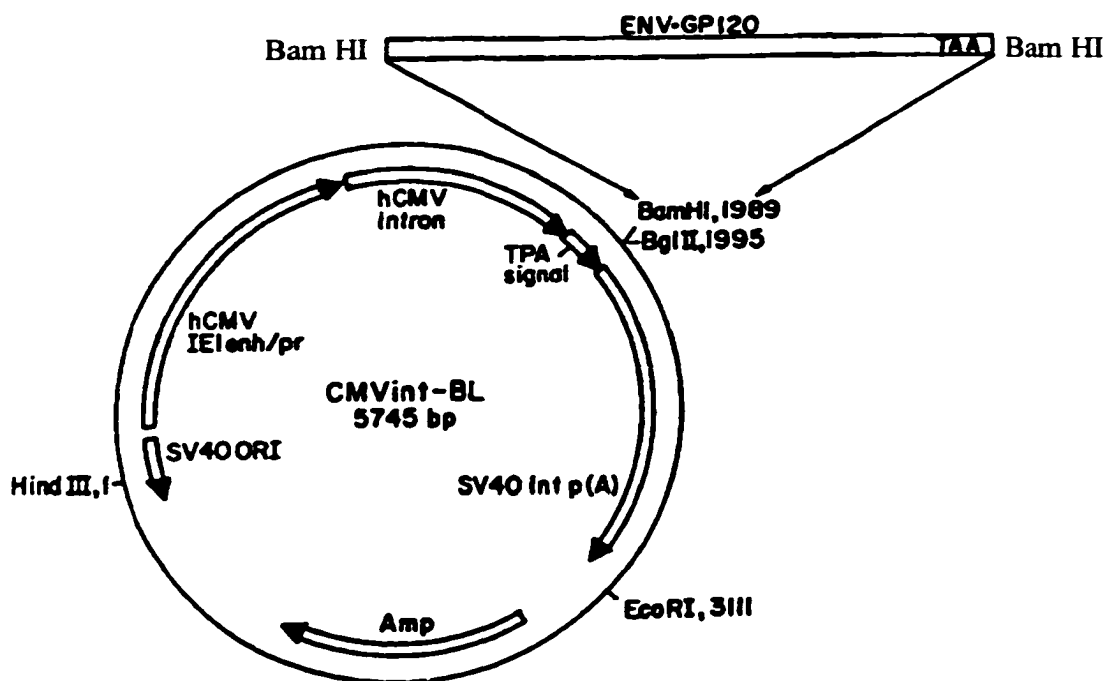


Figure 9. Map of the shuttle vector, CMVint-BL used for immunization. The gp120 insert was ligated at the Bam HI site immediately following the TPA signal sequence. Expression was driven from the TPA start codon (ATG). The stop codon for rgp120 was included in the downstream PCR primer used to amplify ENV (adapted from reference 32).

sequencing primer used to screen for in-frame ligations was designed from the TPA signal sequence in CMVint-BL as follows: 5'-GGCTCTGCTGTGTGCTG. The plasmids were diluted in 0.9 % saline to 4 mg of DNA/ml concentration for use in immunization (following section).

Sequence analysis. DNA sequences used in this study, A-UG06c, B-RT3.12, C-UG045, D-UG042 and D-UG23c, were generated as previously described (32, 35, 36, 45). Predicted amino acid sequences were aligned in CLUSTAL in PC/Gene (version, 6.85; Intelligenetics, CA).

Mutagenesis of D-UG23c → mD-UG23c. Using a unique-site elimination mutagenesis protocol (U.S.E. kit, Pharmacia Biotech, CA), a Leu → Pro substitution was introduced in the apical tetrapeptide (GLGQ → GPGQ) encoded in D-UG23c. The ENV insert in D-UG23c (32) was recovered by Bam HI restriction and then subcloned into the BamH I-digested pUC19 vector. The oligonucleotide primer 5'-TATAGGACCAGGGCAAGCACTC was used to introduce the T → C substitution in the V3-encoding region. The substitution created a new BstN I site which was used for the selection of the mutants. All mutagenesis reactions were performed as described in the manufacturer's protocol. Oligonucleotide phosphorylation was conducted in 30 µl of reaction mix containing 300 pmol of the mutagenesis primer, 10 mM Tris acetate, 10 mM magnesium acetate, 50 mM potassium acetate (pH 7.5), 10 mM ATP and 20 units of T4 polynucleotide kinase. The reaction mix was incubated at 37 °C for 30 min. The reaction was stopped by heating the mixture to 65 °C for 10 min. The mutagenesis reaction was performed in a total volume of 20 µl containing 1 µg of plasmid DNA, 25 pmol of the U.S.E. selection primer (5'-CTGTGACTGGTGACGCGTCAACAAAGTC), 25 pmol of

the target mutagenesis primer (5'-TATAGGACCAGGGCAAGCACTC, Keystone, CA) in a 10 mM Tris acetate (pH 7.5) buffered solution. The U.S.E. selection primer converts the Sca I site (ACGCGT) in pUC19 to a Mlu I site (AGTACT). This mutation allows for selection of the mutated plasmid. The mix was then incubated at 100 °C for 5 min to denature the plasmid, chilled on ice for 5 min and then incubated at room temperature for an additional 30 min to allow annealing of the plasmid and primers. The extension and ligation reaction was carried out by incubation at 37 °C for 1 hr with the addition of 7 µl of nucleotide mix [2.86 mM each dATP, dCTP, dGTP and dTTP; 4.34 mM ATP; 1.43x One Phor-All Buffer PLUS, Pharmacia] and 3 µl of reaction mix [T4 DNA ligase (0.83-1.17 ku/ml), T4 DNA polymerase (0.2 mg/ml)]. The mixture was heated at 85 °C for 15 min to stop the reaction. The first round of screening for successful mutagenesis was conducted by digestion with Sca I (NEB). Thirty microliter aliquots of the mutagenesis reaction was digested with Sca I at 37°C for 2 hr to eliminate the unique Sca I site in the wild type recombinant and to reduce the transforming ability of wild-type plasmids (Fig. 10). Five microliter of digested DNA from the 30 µl mutagenesis reaction mix was then used to transform 200 µl of a repair-defective (mutS) *E. coli* strain, NM522 in 3 ml of LB medium containing 100 µg/ml of ampicillin. Plasmid DNA was prepared from a liquid culture of transformants by the alkaline lysis method (48) for a second round of restriction enzyme screening with Sca I (Fig. 10). Epicurian *E. coli* XL-1 Blue competent cells were used for the second transformation. Plasmid DNA prepared from selected colonies were digested with BstN I (NEB) to ensure that the Sca I positive plasmid had the newly created BstN I site. The introduction of the point mutation was further verified by DNA sequencing as described above. The mutant ENV clone was subsequently cloned

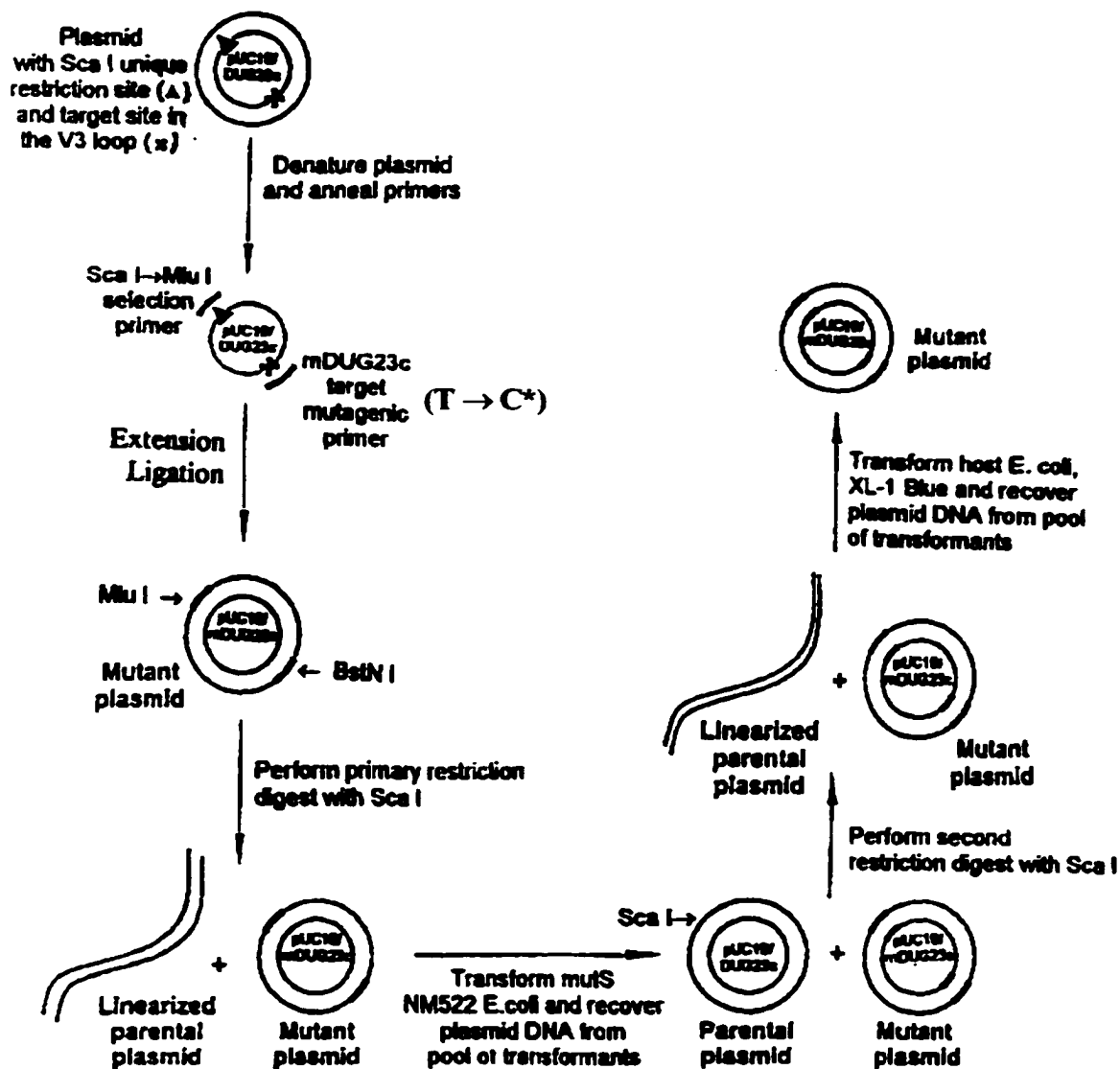


Figure 10. Flow chart of site-directed mutagenesis of the V3 loop in D-UG23c (modified from the manufacturer's diagram, Pharmacia Biotech). The sequences for the selection primer and the target mutagenic primer are described in Materials and Methods.

into the Bam HI site in CMVint-BL vector and used for immunization (32) and designated mD-UG23c.

β-turn analysis. The program BETATURN in PC/Gene was used to predict tetrapeptide sequences which are likely to assure the antigenic β-turn in protein secondary structure conformation (35, 36, 45, 51). BETATURN uses the Chou and Fasman algorithm to identify this motif and its application for the determination of the antigenic regions in gp120 has been previously described (32, 35, 36, 45). It should be noted that the β-turn conformation does not alter binding affinities of peptides to the ELISA plate, instead this motif affects the ability to elicit antibody responses (35, 45, 56).

In vitro transfection assay. Freshly passaged CEM cells were washed with PBS and resuspended at 1×10^6 cells/ml in complete growth medium [RPMI 1640 (GIBCO, NY) containing 10% FCS, 2 mM Glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin and 1 mM non-essential amino acids]. Seventy-five µl of reduced serum medium (GIBCO, NY) were mixed with 2.5 µg of DNA and 15 µl of SuperFect transfection reagent (Qiagen, CA). The mixture was centrifuged for 10 sec and then incubated for 15 min at room temperature. The reagent mix (90 µl) was added dropwise to 2.5 ml of the cell suspension in 30 mm culture plate. The cells were swirled several times and then cultured at 37°C in 5% CO₂ and 95% humidity. The cells were washed 3 × with PBS. The PBS was aspirated, and the cell pellet was stored at -70°C until tested in RT-PCR (following section).

RT-PCR. To assess the in vivo expression of ENV, the DNA-inoculated quadriceps muscle of the mice was dissected and powdered using a pre-chilled mortar

and pestle. The powder (0.4 g) was resuspended in 4 ml of lysis buffer (Boehringer Mannheim, IN) and homogenized. The homogenate (800 μ l) was spun for 30 sec, and 20 μ l of oligo(dt)₂₀ working solution was added. The sample was incubated for 6-9 min at 4°C and 50 μ l of the mix was added to a streptavidin-coated tube and incubated for 6 min at 4°C. The mix was removed and the tubes were washed three times. The RNA samples were then processed for semi-quantitative RT-PCR as described in the mRNA capture and Titan RT-PCR kits (Boehringer Mannheim, IN). The following primers were used for the clones A-UG06c, B-RT3.12 and C-UG045: upstream 5'-TGGGATCCAGTCTAGCAGAAGAAGAG, downstream 5'-TTCGGATCCCTAATTGTCCTCATATCTC; and for the clones D-UG23c and mD-UG23c: upstream 5'-CCTGGATCCCTCACAAACAATGCTA, downstream 5'-CAAGGATCCTCAGAACATAGCTCCTAGTC; and the β -actin PCR primer pair (R&D Systems, MN) was the following: upstream 5'-CTACAATGAGCTGCGTGTGG, downstream 5'-AAGGAAGGCTGGAAGAGTGC. The reverse transcription reaction was performed at 50°C for 30 min followed by one cycle at 94°C for 5 min. PCR was performed as follows: denature (95°C) 30 sec, anneal (50°C) 30 sec, extend (72°C) 1 minute for 35 cycles, and a final extension at 72°C for 10 min. The PCR products were analyzed to determine the relative gene expression levels as previously described (52).

DNA immunization. Groups of 6-week-old BALB/c mice (4-6 mice per group) were inoculated in the quadriceps muscle with 100 μ g of the plasmid in 200 μ l of saline using a 1cc tuberculin syringe and 28G needle as previously described (32). The animals were boosted with the same dose of DNA at two-week intervals. Control mice were inoculated with CMVint-BL (empty vector, no ENV). Serum samples were obtained

from 200-400 μ l of blood collected from the tail vein after two weeks of boost (Fig. 11). To recover sera, blood samples were allowed to clot at 4°C overnight and then centrifuged at 10,000 rpm for 10 min. The serum specimens were preserved at -20°C until tested.

ELISA. Homologous peptides comprising the predicted V3 epitope and CD4 binding site were synthesized from the deduced amino acid sequence for each of the ENV clones. The methods of prediction, synthesis and characteristics of these linear B cell epitopes have been described elsewhere (35, 36, 45). Peptides comprising the V3 loop (V3) and the linear CD4 binding region (CD4 site) were synthesized (Table 2) and purified as previously reported (35, 45). Duplicate assays were conducted using the V3 and CD4 site peptides (Table 2). The assay was performed essentially as described (53). These studies had previously indicated that the use of 125 μ l per well of test peptide at a concentration of 50 μ g/ml in NaHCO₃ buffer (pH 9.6) offered the most discrimination (> eightfold difference) between the test and control sera when used at a 1:50 dilution or lower. Ninety six-well flat-bottomed PVC microtiter plates (Falcon, CA) were sensitized by overnight incubation at 4 °C with 125 μ l of the respective peptides at a concentration of 50 μ g/ml in 0.1 M NaHCO₃ (pH 9.6). The solution was discarded. The plates were blocked by incubation with 125 μ l of 1 % BSA (Boehringer Mannheim) in PBS [137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄] (pH 7.4) for 1 hr and 20 min at 4 °C. After one wash with 150 μ l of PBSTB [PBS containing 0.05 % Tween 20 (Sigma) and 1 % BSA], 125 μ l of the test serum (diluted 1/50 in PBSTB) was added. The plates were incubated for 1 hr and 20 min at 4 °C. The wells were washed 10 times with 200 μ l of PBSTB and then incubated for 1 hr at 4 °C with 125 μ l (33 ng/ml) of

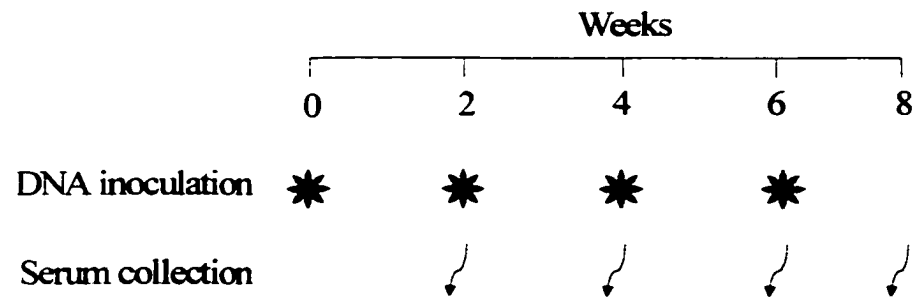


Figure 11. Schedule of immunization and serum collection. Each plasmid was inoculated every 2 weeks post-priming and serum was obtained after two weeks of boost.

Table 2. Predicted epitopes in the V3 loop and CD4 binding site tested for immunoreactivity. Symbols in consensus sequence: conserved (upper case); well-conserved (lower case); variable position (?); gaps introduced to facilitate alignment (-); V3 cap (boldface). Peptides underlined were synthesized.

	V3 loop	CD4 site
Consensus	CtRP?nn?r???hi GpGq afytt??iigdIrqA?C	?VGkaMYAPPI?G?ItC?S
A-UG06c	CTRPYKKVRRRKHI GPGRS FYTSN--IGDIRQAYC	
B-RT3.12	CTRPSNNTSKGIHI GPGRA FYTTEAITGDIRRAYC	<u>EVGKAMYAPPIKGQIRCSS</u>
B-MN	CTRPNYNKRKRIHI GPGRA FYTTKNIIGTIRQAHC	
C-UG045	CTRPNNNTRESVRI GPGQ AFYATKDIIGDIRQAHC	<u>GVGRAMYAPPIEGKITCKS</u>
D-UG23c	CTRPYENVRHRTPI GLGQ ALITNR- <u>IKAKIGQAYC</u>	<u>RVGKAMYAPPIQGKITCVS</u>
mD-UG23c	CTRPYENVRHRTPI GPGQ ALITNR- <u>IKAKIGQAYC</u>	
D-UG042	CTRPYSKIRQRTST GQGQ ALY-TTKGRGIIGQAYC	

Symbols: Consensus: Identical residues (uppercase); well-conserved (lowercase); variable (?); apical V3 residues (bold-faced).

Alignment: Synthesized peptides (underlined); gaps to facilitate alignment (-).

horseradish peroxidase-labeled goat anti-mouse whole IgG (Promega, WI) diluted in PBSTB. After 10 more washes with PBSTB, antibody binding was detected by the addition of 125 μ l of OPD (o-phenylenediamine dihydrochloride) substrate buffer containing 1 tablet of each OPD (Sigma) and urea H₂O₂ (Sigma) in 20 ml of H₂O. The reactions were stopped by the addition of 50 μ l of 4 M H₂SO₄. O.D. readings were determined at 490 nm using a microplate reader (model 550, BIO-RAD, CA). Values from duplicate assays varied by less than 5 %. Sera from the control mice inoculated with CMVint-BL only were analyzed simultaneously to provide background reactivities.

Proliferation assay. Splenocytes were recovered from the experimental or control mice by teasing spleens in RPMI 1640 culture medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine, 20 mM HEPES and 5×10^{-5} M β -mercaptoethanol. The splenocytes were washed and then adjusted to 1×10^6 cells/ml. Two hundred μ l of the cell suspension were stimulated in triplicate with synthetic peptides (25 μ g/ml) in 96-well microculture plates for three days at 37°C in 5% CO₂ and 95 % humidity. The cells were then pulsed for 4 hr by the addition of 1 μ Ci of ³H-Thymidine (³H-Tdr, DuPont, DE). Radiolabeled DNA was harvested on glass fiber filter paper and then quantified using a liquid scintillation counter (WALLAC 1410, Pharmacia).

Measurement of IL-2 secretion. Splenocytes from immunized mice were recovered and spontaneous release of IL-2 (54) was determined after 48 hr of culture using a commercially available ELISA kit (EM-IL-2 ELISA kit, Endogen, MA). ELISA was performed in duplicate for each test sample. O.D. readings were determined at 450

nm and the mean of values was calculated. Student “t” test was used to compare the levels of cytokine secreted in culture.

Intracellular cytokine assay. Intracellular cytokine assay was performed as described (55) with modifications for mouse spleen cells. PMA and anti-CD3 monoclonal antibody was used to restimulate splenocytes recovered from in vivo-primed animals for intracellular cytokine detection prior to FACS analysis. Splenocytes were adjusted to 3×10^6 cells in 2 ml of RPMI complete culture medium containing 10 μ M brefeldin A (Roche, IN), 1ng/ml of PMA (Sigma, MO), and 5 μ g/ml of anti-CD3 monoclonal antibody (PharMingen, CA) in 24-well culture plate. After incubation for 18 hours, the cells were harvested and resuspended in 100 μ l FACS buffer (55). Cells were then stained with 1 μ g anti-CD4 PE conjugated antibody and 1 μ g anti-CD8 Cy5 conjugated antibody (PharMingen) for 30 min at 4°C followed by washing with 1 ml of FACS buffer. For cytokine staining, cells were permeabilized using the FIX and PERM kit (Caltag, CA) according to the manufacturer’s protocol. Fixed cells were incubated for 15 min at room temperature with 1 μ g FITC-conjugated anti-cytokine antibody (IL-2 or INF- γ , PharMingen). Finally, the samples were washed with 5 ml PBS and resuspend in 500 μ l of FACS buffer containing 2% para-formaldehyde for FACS analysis (ELITE, Coulter, FL).

RESULTS

Sub-cloning of ENV into CMVint-BL. The truncated ENV genes for B-RT3.12, C-UG045 and D-UG23c and full length gp120 for A-UG06c were amplified (Fig. 12) and respectively inserted at the Bam HI site in CMVint-BL (Fig. 9). In-frame ligation and the orientation were confirmed by restriction analysis with Bam HI and direct sequencing. The agarose gel shows linear CMVint-BL: 5.7 kb and ENV inserts: 625 bp for B-RT3.12, C-UG045 and 725 bp for D-UG23c (Fig. 13). Ligation of A-UG06c into CMVint-BL vector eliminated one Bam HI site, resulted in 7.3 kb single band (Fig 13). The CMVint-BL constructs of the ENV gene from A-UG06c, B-RT3.12, C-UG045, D-UG23c D-UG042 and mDUG23c were, respectively, designated CMV/A-UG06c, CMV/B-RT3.12, CMV/C-UG045, CMV/D-UG042 and CMV/D-UG23c. Site-directed mutagenesis of D-UG23c resulted in the production of mD-UG23c which was sub-cloned into CMVint-BL and then designated CMV/mD-UG23c.

Time course transcription of gp120 mRNA. Freshly passaged CEM cells were transfected with the respective ENV plasmids using a liposome-mediated protocol. The accumulation of rgp120 mRNA was monitored using the RT-PCR procedure (Fig. 14). The patterns of transient in vitro transcription of all plasmids described in this study were essentially the same as those presented for the clones A-UG06c, B-RT3.12 and C-UG045 (data not shown). The in vitro accumulation of rgp120 transcripts was detected by 24 hr post lipofection and persisted for at least 7 days, at which point the cultures were terminated due to cell crowding and deterioration. This declining trend of expression would be expected since none of the plasmids was designed to integrate into chromosomal

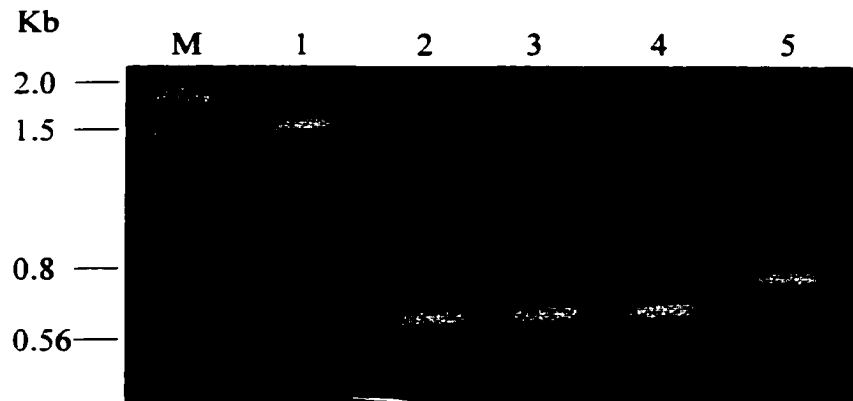


Figure 12. Agarose gel showing PCR products from ENV: A-UG06c (lane 1), B-RT3.12 (lane 2), C-UG045 (lane 3), D-UG042 (lane 4) and D-UG23c (lane 5); Marker DNA (M). The PCR products were analyzed in 1 % agarose gel.

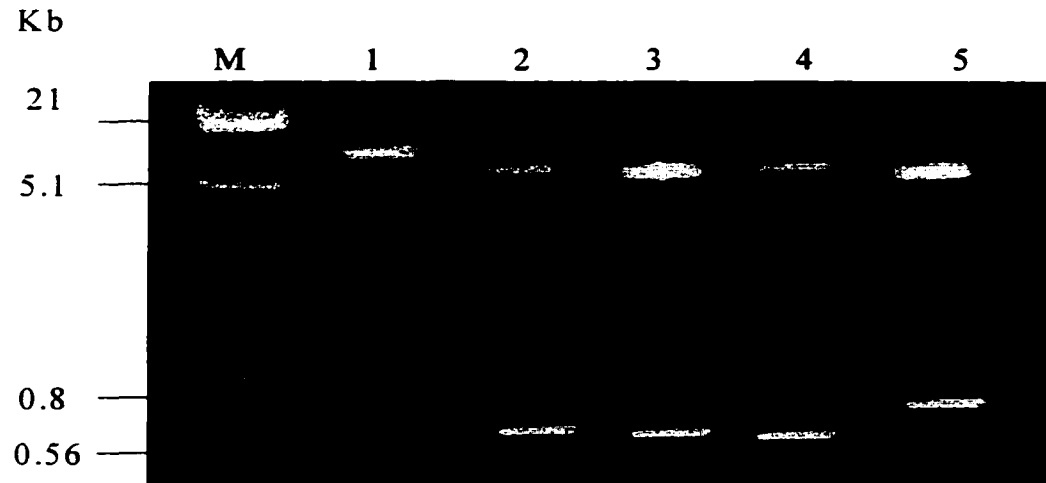


Figure 13. Bam HI restriction analysis of ENV plasmids: CMV/A-UG06c (lane 1), CMV/B-RT3.12 (lane 2), CMV/C-UG045 (lane 3), CMV/D-UG042 (lane 4) and CMV/D-UG23c (lane 5). Marker shown (M). The DNA samples were resolved using a 1 % agarose gel. Lane 1 shows one single band containing CMVint-BL vector and ENV insert (7.3 kb).

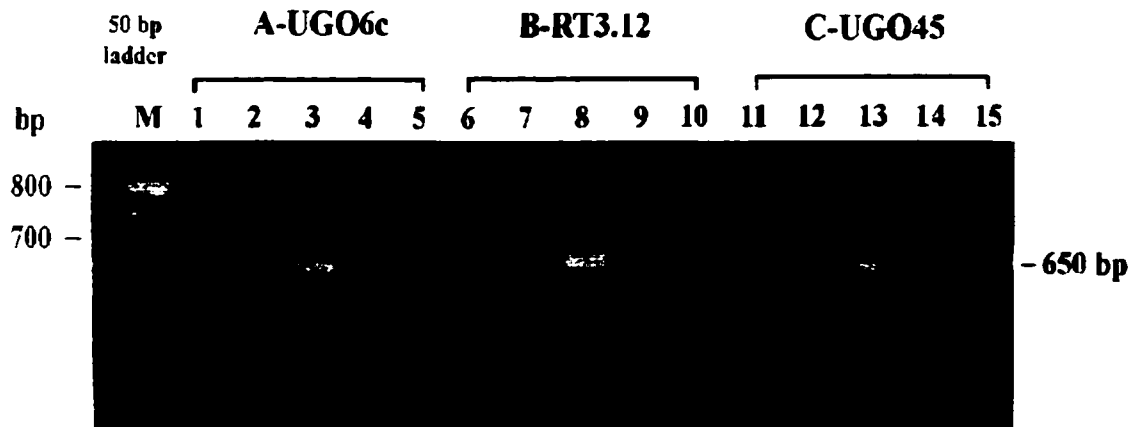


Figure 14. Time course of in vitro transcription of *rgp120* mRNA detected by RT-PCR. Transcription levels on days 1, 3, 5 and 7 are shown, respectively, for clones CMV/A-UG06c (lanes 2-5), CMV/B-RT3.12 (lanes 7-10), and CMV/C-UG045 (lanes 12-15); RT-PCR performed on same mRNA preparations in absence of reverse transcriptase (lanes 1, 6, and 11). A similar pattern of expression was recorded for the clones CMV/D-UG042 and CMV/D-UG23c (data not shown). Marker shown (lane M).

DNA or to replicate autonomously. Other studies have previously indicated that much of the plasmids which is injected into muscle is degraded within 1-2 weeks, but that a minor proportion of the molecules persist and appear to serve as a continuous source of antigen in DNA-mediated immunization. The results shown herein support that observation.

Region of rgp120 analyzed. The region of gp120 encoded by these plasmids range from 192 to 488 amino acids in length (Table 3). Specifically, the clones B-RT3.12, C-UG45, D-UG042 and D-UG23c encode the C2-V5 domain of gp120 (32, 35, 36). This segment comprises the previously defined predominant antibody neutralizing determinant located in the V3 loop, the CD4 binding site and at least six other potential B cell epitopes including an “immunodominant” region close to the gp120/41 fusion site (32, 35, 36, 45). The clone A-UG06c encodes the full-length gp120 molecule (32). In this study I have analyzed the ability of specific V3 variants to elicit humoral and cell-mediated responses.

Divergent clones of rgp120 induce different levels of antibody response to the V3 loop. Inoculation of the ENV plasmids into the quadriceps muscle of BALB/c mice presumably resulted in the expression of rgp120. These gp120 molecules elicited highly variable levels of antibody response as detected by ELISA (Fig. 15). Serum antibody reactivity was tested using synthetic peptides homologous to the previously defined neutralizing epitope in the V3 loop (51, 54, 41). These results apparently identified two different subsets of rgp120 encoded by the plasmids based on their immunogenic potential for B cells. One set of the molecules expressed by the clones A-UG06c, B-RT3.12 and C-UG045 elicited high levels of antibodies to the V3 loop, but the mice

Table 3. Predicted amino acid sequence encoded by the ENV clones ligated into CMVint-BL for DNA immunization. Symbols: gaps introduced to facilitate alignment (-); V3 cap (boldface). Domain assignment in gp120 is shown (C1-C4) according to Modrow et al. (33). The sequence of the subtype B clone MN retrieved from GenBank, was used as a reference clone for comparison.

		-----C1----->	
A-00140	ISAAEDLWVTVVYGVVWKADETTLPCASDAKAYDTEVHNWATHACVPTDPNPQELNGLVTEDFNMWKNMVEQMHEDIISLWQGLKPCVWLTPLCV	100	
B-MN	ISAAEKLWVTVVYGVVWKAETTLPCASDAKAYDTEVHNWATQACVPTDPNPQEVLELVNVTENFMWKNMVEQMHEDIISLWQGLKPCVWLTPLCV	100	
		-----V1-----> <-----V2-----> <-----C2----->	
A-00140	TLNNTLAKAVVNASMTSASINNTGGM--QGEIKNCSFNMTELRCKTKQKYSLFYKLVVGINENNGSTNNTGSGQYRLIHCSTSTITQACPKVTFEP:	196	
B-MN	TLNNTLAKAVVNASMTSASINNTGGM--QGEIKNCSFNMTELRCKTKQKYSLFYKLVVGINENNGSTNNTGSGQYRLIHCSTSTITQACPKVTFEP:	196	
		-----C3-----> <-----V3----->	
A-00140	PIHYDAPAGFALDKNDSEFNGTGCEKRVSTVQCTHGIRPVVSTQLLLNGSLAEKEVYIRSENI SDNAKTIIVQLTKF-VTINCT P FYKIVPFRKHI--G	296	
B-MN	PIHYDAPAGFALDKNDSEFNGTGCEKRVSTVQCTHGIRPVVSTQLLLNGSLAEKEVYIRSENI SDNAKTIIVHLNES-VGINCTRPNYKRRKRLHI--G	299	
B-00140		SLAEKEVYIRSENI SDNAKTIIVQLNKS-VEINCT RPSNNTSKGIHI--G	47
D-00140		SLAEKEVYIRSENI SDNAKTIIVHLNES-VEINCT RPNNTRESVRI--G	47
D-00140		ENLTNNAKTIIVQLNET-VTINCT RPYENVRHPTPI--G	36
HL-00140		ENLTNNAKTIIVQLNET-VTINCT RPYENVRHPTPI--G	36
D-00140		SLAEKEVYIRSENI SDNAKTIIVQLNET-VGINCT RPYENVRHPTPI--G	47
		-----V3----->	
A-00140	PGRSFI-TSNI-GDIRQAYCNVSGSQWNTLQQVYTKLREYWN-TTINFTK-PSGGDLE-IITHSFNC-GGEFFYCNTSGLFNSSW----KI--GSQPN	362	
B-MN	PGRAFYTTNIIGTIRQAHONI SRAKWN DTLRQIVSKLKEQF-KNKTIVENQSSGGDPE-IGMHSFNC-GGEFFYCNTSPLFNSTWNGHNTWN----RRT	362	
B-00140	PGRAFYTTNIIGTIRQAHONI SRAKWN DTLRQIVSKLKEQF-KNKTIVENQSSGGDPE-IGMHSFNC-GGEFFYCNTSPLFNSTWNGHNTWN----RRT	140	
D-00140	PGQAFYATKDIIGDIRQAHCHNNGTKWNATLQKVRKLRQYFFD-RTIEFNNSAGGDLE-IITHSFNCRG-EFFYCNTSGLFNST-----	109	
D-00140	LGQALI-TNFIRKAKIGQAYCNI SKTEWQKTLQPVATKLRDLLNHTTINFKP-SGGDQE-IITHSFNC-RGEFFSCNTSKLFNSTW----NS--TSNST	125	
HL-00140	PGQALI-TNFIRKAKIGQAYCNI SKTEWQKTLQPVATKLRDLLNHTTINFKP-SGGDQE-IITHSFNC-RGEFFSCNTSKLFNSTW----NS--TSNST	125	
D-00140	QQQALI-TTKGRDIIGQAYCNI SGAENKTLHSSNI-LRFPFSQTRINLPHPLEGTPE-IITHSFNC-GGEFFSCNTSKLFNSTW----NS--GTLNG	136	
		-----V4-----> <-----C3-----> <-----V5-----> <-----C4-----> -gp120// -	
A-00140	SHESNGNITLPCRIRQIIRMQRTGQA-TYAPPIQGIVRCESNITGLQLTRDGG--VNST--ETFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPFRA	476	
B-MN	IQHNN--ITLPCRIRQIIRMQRTGQA-TYAPPIQGIVRCESNITGLQLTRDGG--VNST--ETFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPFRA	477	
B-00140	TA-CDGKFI LPCRIRQIIRMQRTGQA-TYAPPIQGIVRCESNITGLQLTRDGG--VNST--ETFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPFRA	211 a.a.	
D-00140	---IDPNI LPCRIRQIIRMQRTGQA-TYAPPIQGIVRCESNITGLQLTRDGG--VNST--ETFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPFRA	192 a.a.	
D-00140	DIACKT-IRLPCRIRQIIRMQRTGQA-TYAPPIQGIVRCESNITGLQLTRDGG--VNST--ETFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPFRA	216	
HL-00140	DIACKT-IRLPCRIRQIIRMQRTGQA-TYAPPIQGIVRCESNITGLQLTRDGG--VNST--ETFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPFRA	214	
D-00140	PKLNIITLPCRIRQIIRMQRTGQA-TYAPPIQGIVRCESNITGLQLTRDGG--VNST--ETFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPFRA	209 a.a.	
		-pp41--511-	
A-00140	FFRNVVEREFA	466 a.a.	
B-MN	FFRNVVEREFA	469 a.a.	
D-00140	FFRNVVEREFA	230 a.a.	
HL-00140	FFRNVVEREFA	230 a.a.	

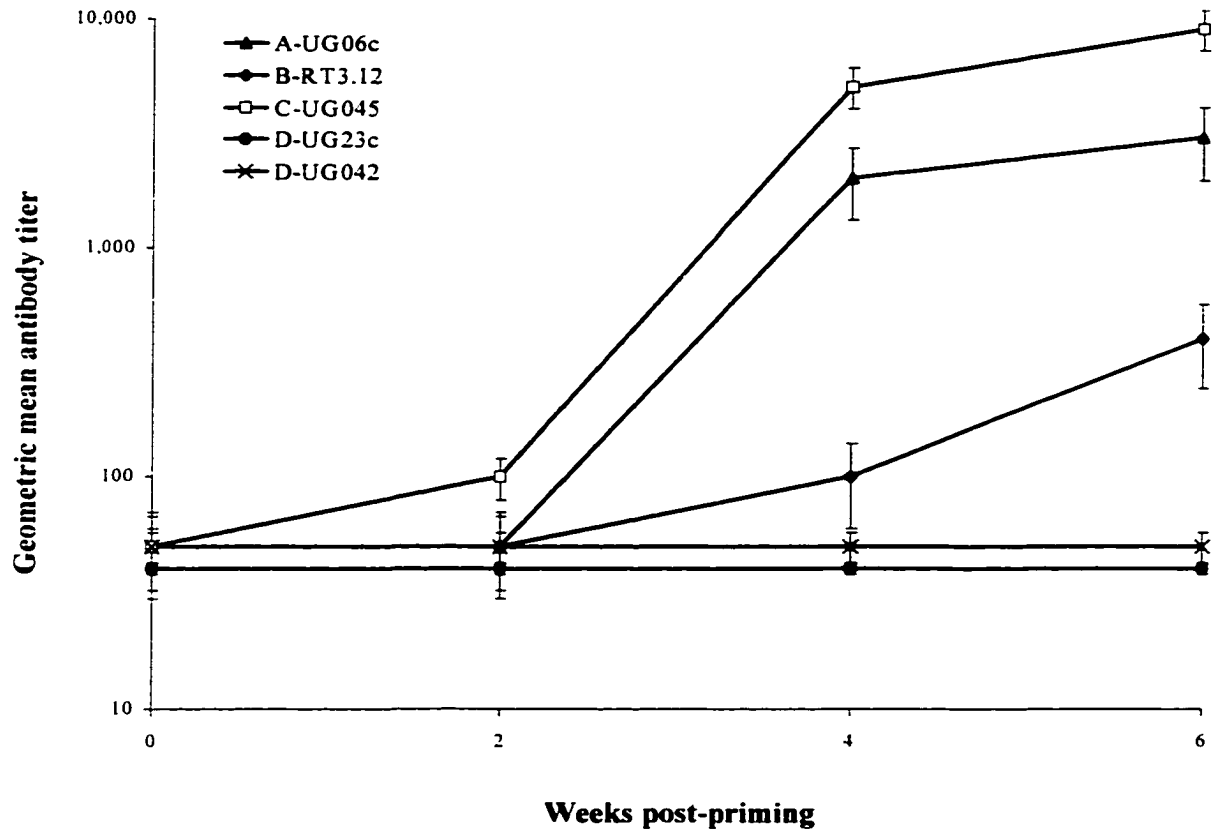


Figure 15. ELISA titers of anti-V3 loop antibodies induced after mouse immunization with different ENV plasmids. Each point represents the mean titer of antibodies of the respective group of mice that responded to immunization. Antibody reactivity was assayed against the homologous V3 peptide. Error bars (SD) shown are for groups of 4 mice tested at each time point.

inoculated with the other subset of plasmids, D-UG23c and D-UG042, showed no evidence of antibody production to this region of gp120.

rgp120 is expressed in mice by the clone D-UG23c. D-UG23c is a prototype of ENV clones that failed to stimulate the production of anti-V3 loop antibodies upon inoculation into mice. I designed experiments to test whether this clone was expressed in vivo, and whether the defect in the induction of V3 antibodies affected all major epitopes found in the encoded gp120 antigen. These experiments demonstrated that inoculation with D-UG23c elicited a strong antibody response to the CD4 binding site after the 6th boost (Fig. 16). The CD4 binding peptide tested in ELISA in this experiment was synthesized based on our previously published sequence data (32, 35, 36, 45). Under the conditions where the D-UG23c clone generated antibody response to the CD4 binding peptide, this clone again failed to elicit a response to the homologous V3 peptide. It seemed unlikely that the V3 peptide derived from D-UG23c was defective as a result of an error in chemical synthesis; it was observed that the homologous V3 peptides synthesized in identical condition for the clones A-UG06c, B-RT3.12 and C-UG045 reacted well with the respective antibodies. The results presented in this set of experiments (Fig. 15) provided the first evidence that a defect in the immunogenic potential of rgp120 encoded by D-UG23c is unique to the V3 loop.

Lack of a β -turn conformation in V3 loop of D-UG23c is associated with a defect in immunogenicity. Although the high level of genetic variability in the V3 loop is widely recognized, the extent to which mutations that occur in the loop alter the antigenic properties of this region of gp120 is relatively unclear. We used the program BETATURN algorithm to analyze the antigenicity of selected V3 sequences from HIV-1

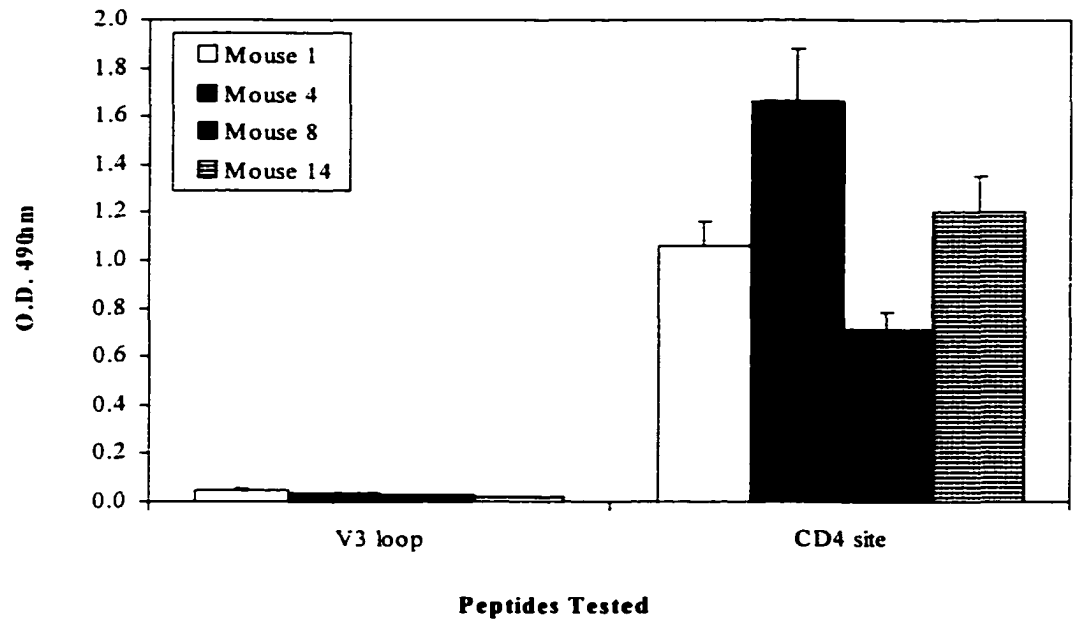


Figure 16. ELISA reactivity of antibodies directed at V3 loop and CD4 binding site expressed by D-UG23c. Individual mice shown were immunized with the D-UG23c plasmid. Sera from the responding mice were tested using synthetic peptides of the homologous V3 loop (Materials and Methods) and CD4 binding site. Mice were bled two weeks after the 6th boost. Error bars are shown for duplicate assays.

field isolates representative of five of the global repertoire, previously identified in our laboratory (35, 36, 45). This analysis indicated that *rgp120* encoded in D-UG23c is unlikely to adopt a β -turn conformation in the V3 loop just as previously reported for the clones D-UG042 and B-RT1 (33, 36, 45). To test whether this defect may alter the antigenic properties of *gp120*, I performed a site-directed mutagenesis assay in which a single codon mutation CTA \rightarrow CCA (GLGQ \rightarrow GPGQ) was introduced into the region of the V3 loop encoded in D-UG23c. The mutation introduced into the V3 region of D-UG23c created a new BstN I site in that plasmid. Restriction analysis with BstN I was conducted to screen transformants for the mutant clone (Fig. 17). The 650 bp of DNA band in mutated D-UG23c was thus cleaved due to this alteration (Fig. 17). DNA sequencing conducted to confirm the mutation in the V3 apex of D-UG23c indicated that this region was successfully mutated from Leu \rightarrow Pro (Fig. 18). The mutated plasmid was designated mD-UG23c. Using β -turn analysis, it was predicted that the change to proline at this position would confer a strong β -turn conformation in the V3 loop of mD-UG23c comparable to that of the immunogenic clones C-UG045 and B-RT3.12 as shown (Fig. 19).

GLGQ \rightarrow GPGQ point mutation significantly enhances humoral response to V3 loop. The clone D-UG23c is apparently a prototype of ENV clones that fail to stimulate the production of anti-V3 loop antibody after immunization. We then designed experiments to assay whether the β -turn reconstituted mD-UG23c and the wild type D-UG23c would differ in their immunogenic properties. Comparable levels of mRNA expression were detected for wild type D-UG23c and mD-UG23c at 5 days post-

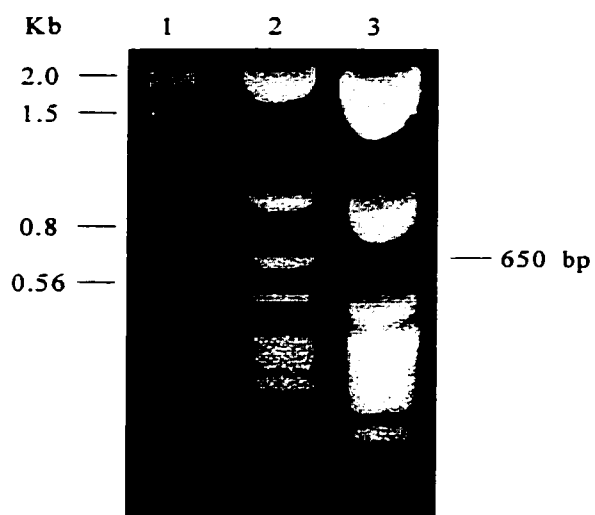


Figure 17. *BstN I* digestion screen for CMV/D-UG23c (lane 2) and CMV/mD-UG23c (lane 3); DNA marker shown (lane 1).

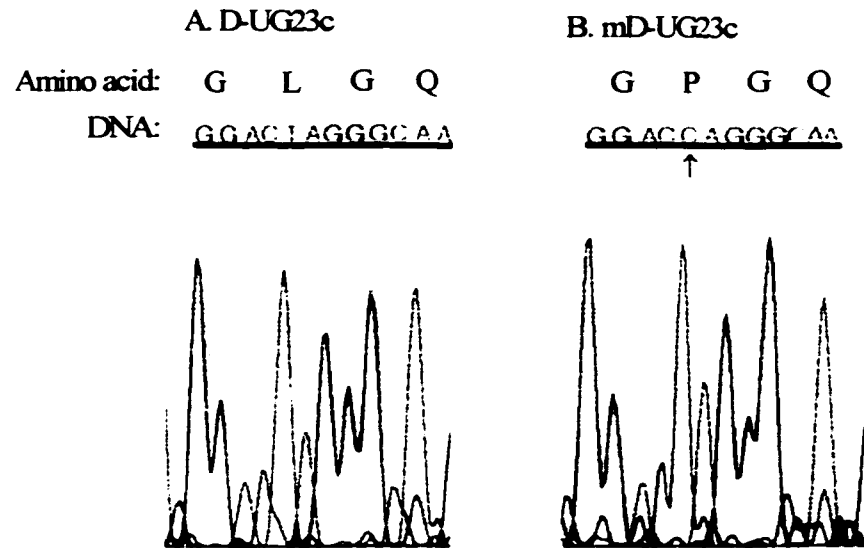


Figure 18. Partial sequence of the V3 loop region in wild type CMV/D-UG23c (A) and mutated CMV/mD-UG23c (B) V3 region. Symbol (↑) indicates mutated nucleotide. The predicted amino acid sequences are shown.

Figure 19. The effect of site-directed mutagenesis (GLGQ → GPGQ) on the probability of a β -turn conformation in the V3 loop encoded by D-UG23c and mD-UG23c. β -turn conformation of immunogenic clones, C-UG045 and B-RT3.12 is shown. β -turn prediction was performed according to Chou and Fasman algorithm (51) in PC/Gene. Bold, underlined residues at the V3 apex. Inverted arrowhead (\blacktriangledown) indicates tetrapeptides (underlined) most likely to assume the β -turn conformation.

Figure 19

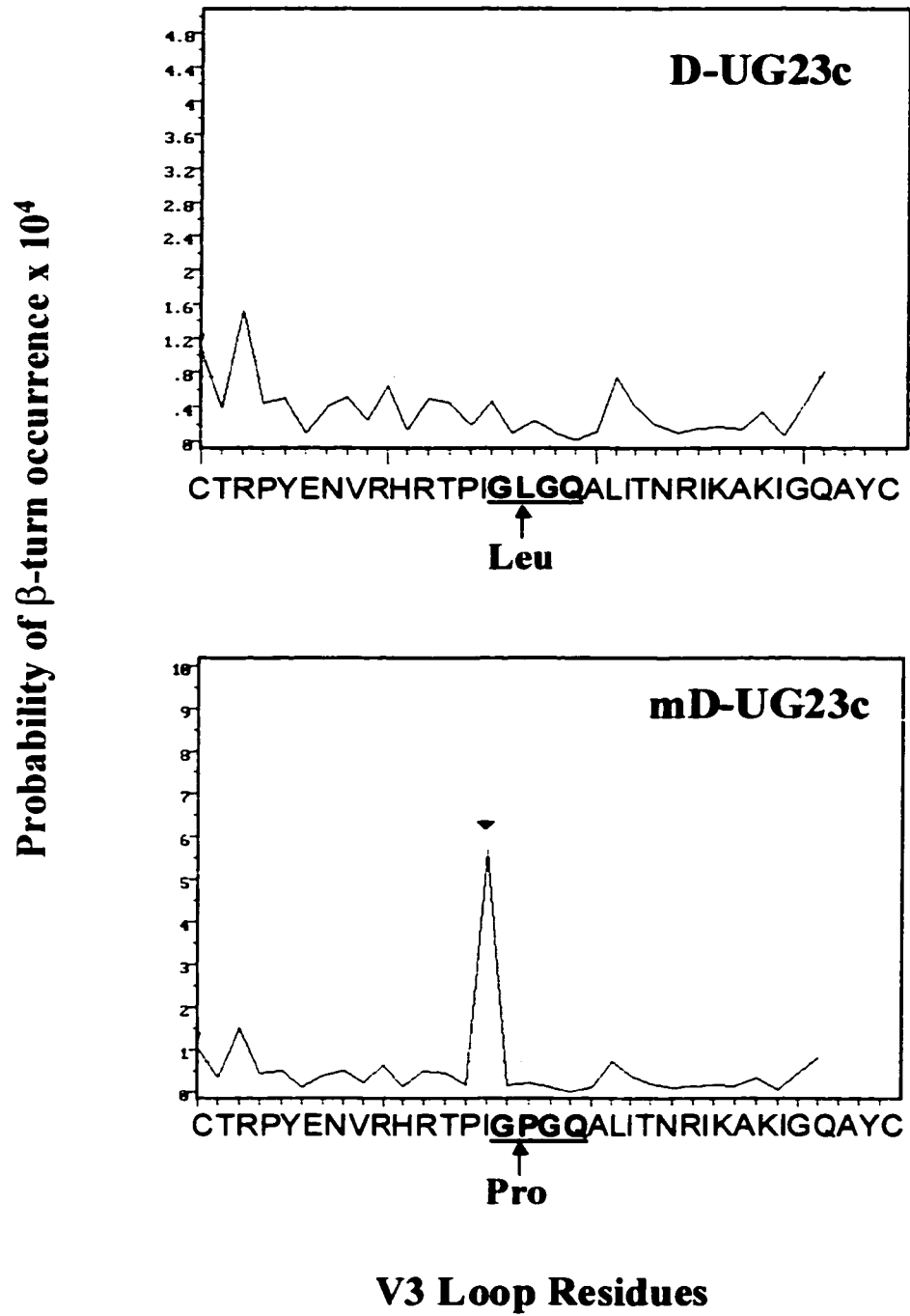
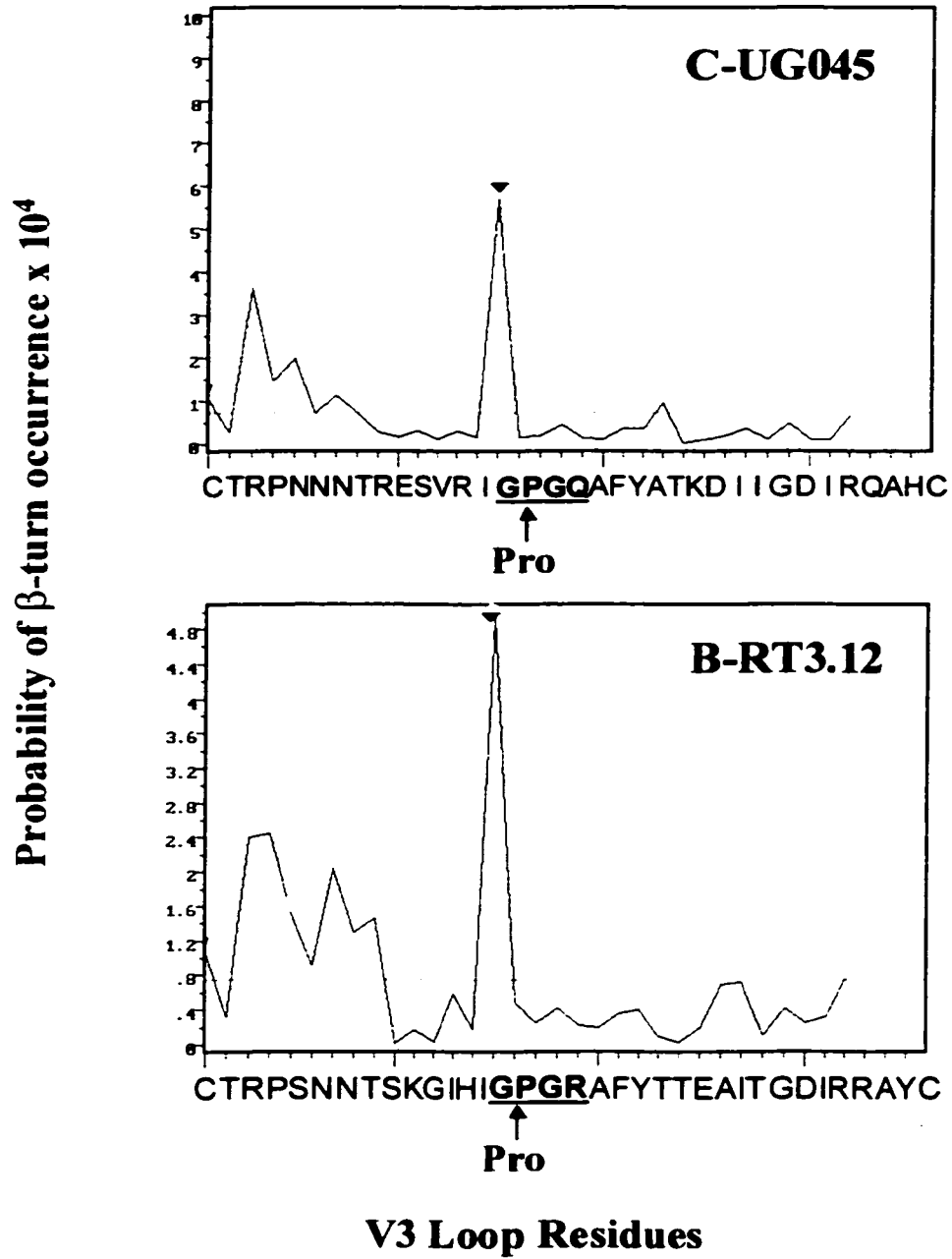


Figure 19



inoculation into the quadriceps muscle of mice as determined by semi-quantitative RT-PCR (Fig. 20). In contrast, it was observed that only the β -turn reconstituted mD-UG23c elicited appreciable levels of V3 loop antibodies in inoculated mice (Fig. 21).

Interestingly, antibodies elicited by immunization with mD-UG23c, also cross reacted with peptides expressing the homologous apical tetrapeptide GPGQ such as C-UG045 (Fig 21). In the second experiment it was observed that only mD-UG23c elicited appreciable titers of V3 antibodies in inoculated mice (Fig. 20). Significantly, immunization with mD-UG23c resulted in the development of antibodies which react with both the homologous and wild type V3 peptide (Fig. 21). Further to test for the impact of β -turn in V3 region, multiple antigen peptides (MAP) from D-UG23c or mD-UG23c were synthesized (Materials and Methods) and immunized into mice for antibody development. The sera from mice immunized with mD-UG23c MAP generated strong antibody to the V3 loop ($n=5$, $p < 0.0033$ as compared to mice immunized with D-UG23c) (Fig. 22). However, MAP comprising V3 loop from D-UG23c showed little evidence of antibody induction. These findings support the concept that β -turn conformation in the V3 loop affects the immunogenicity of HIV-1 gp120.

Selective induction of CMI responses by β -turn deficient rgp120. The results presented above support the view that the ENV D-UG23c is efficiently expressed in mice after immunization. Since TH1 responses may be characterized by T cell proliferation, IL-2 and IFN- γ production as well as CTL responses and Ig isotype production (19, 57, 58), we conducted a variety of assays to determine whether D-UG23c would trigger other form of immune response. T cell proliferation assay was performed on spleen cells of mice that had been previously hyper-immunized with D-UG23c (Fig. 23). Re-stimulation

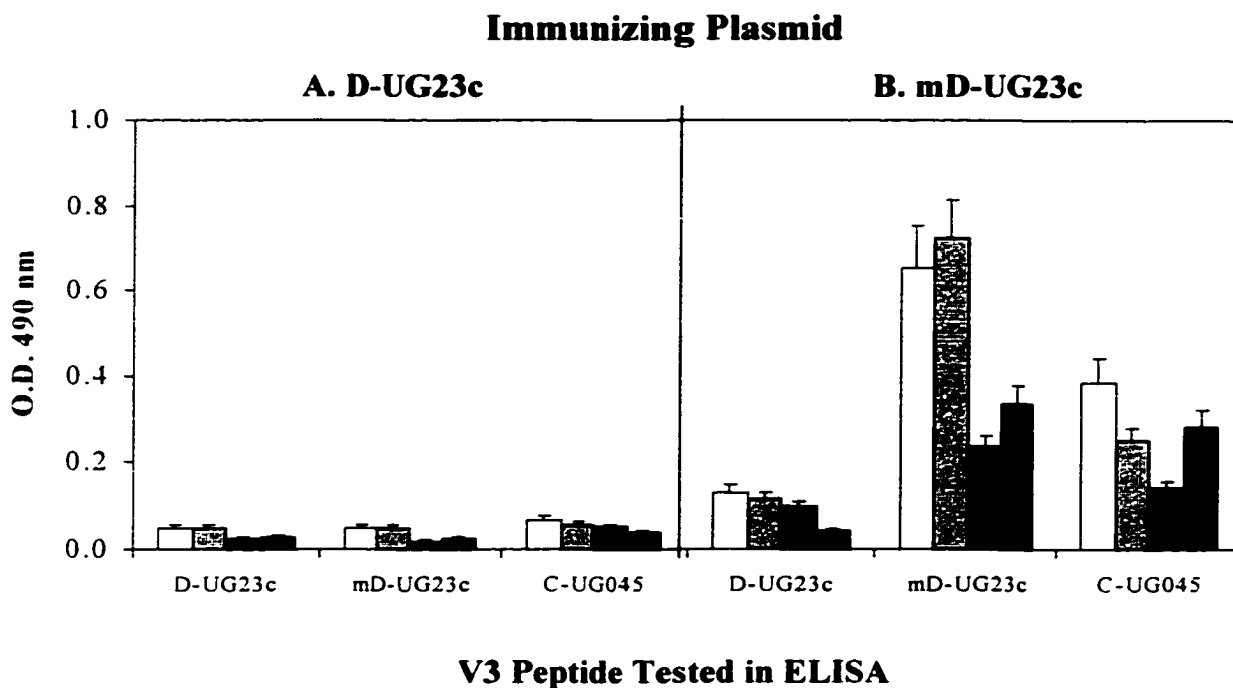


Figure 21. ELISA reactivity of V3 loop antibodies elicited in mice by immunization with the plasmids: (A) CMV/D-UG23c and (B) CMV/mD-UG23c cDNAs. Antibody reactivity was tested against the homologous and divergent V3 peptide after the 4th boost in each experiment. Error bars (SD) are shown for duplicate assays conducted on sera of 4 representative individual mice shown by the different shaded bars within each panel. The results shown are representative of 20 mice immunized with either clone.

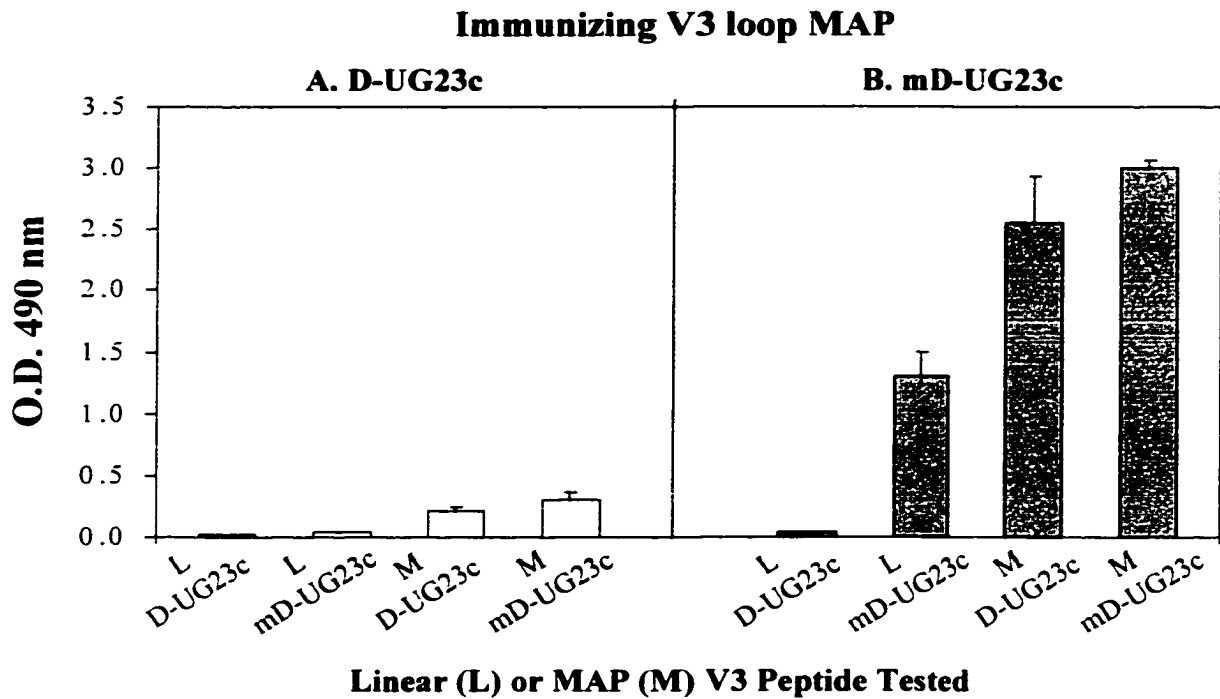


Figure 22. Reactivity of antibodies from mice immunized with multiple antigen peptides (MAP) comprising V3 loop of each clone, A: D-UG23c; B: mD-UG23c. Sera were tested against linear or MAP V3 peptide after the 2nd boost in each experiment. Data shown are the mean and error bar (SD) for 5 individual mice. Linear peptide (L); MAP peptide (M).

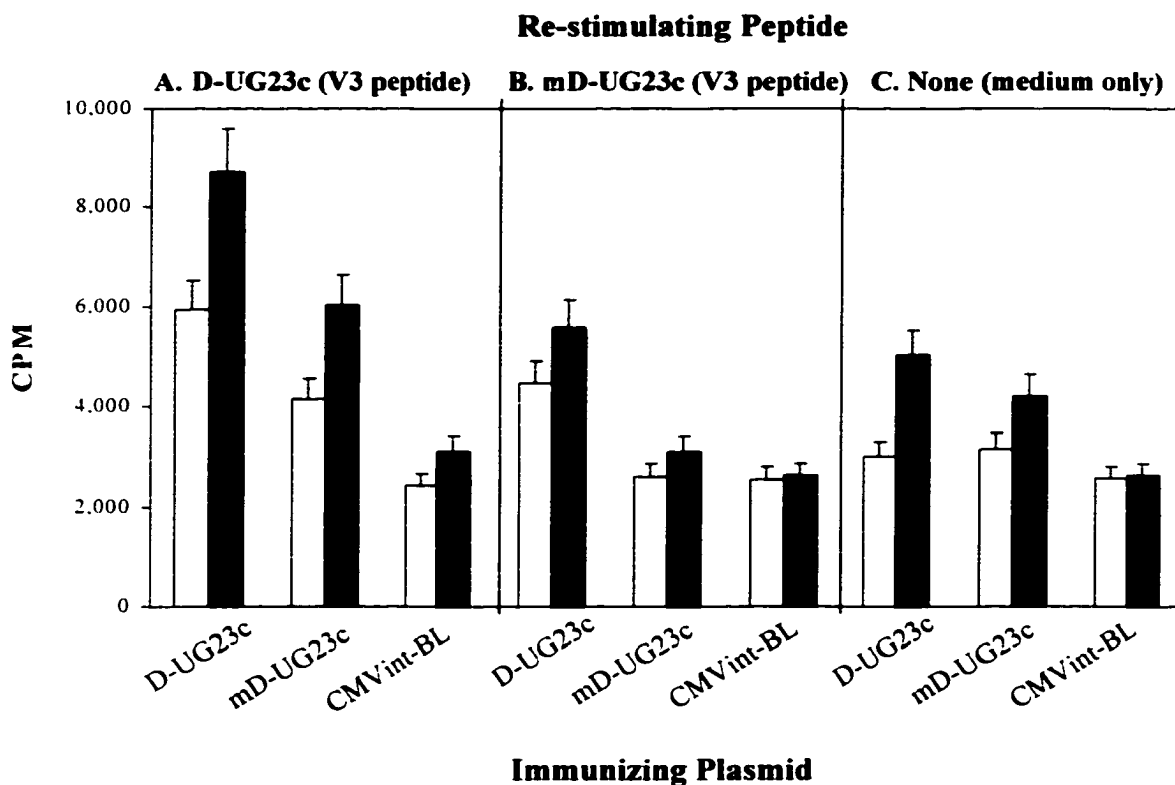


Figure 23. Proliferation (^3H -Tdr incorporation) of spleen cells of mice immunized with CMV/D-UG23c or CMV/mD-UG23c. Splenocytes were re-stimulated *in vitro* with the respective V3 peptides of the clones (A) D-UG23c, (B) mD-UG23c or (C) medium only after the 4th boost (Materials and Methods). Each value shown indicates the mean and standard deviation of triplicate assays. Data shown are two representative experiments (unshaded and shaded bars) of five different experiments.

of splenocytes with the homologous V3 peptide selectively boosted the T cell proliferation (Fig. 23). In contrast, negligible T cell proliferation was observed for cells recovered from mice immunized with the mutant clone, mD-UG23c. These results demonstrate the selective ability of D-UG23c to induce CMI response to the V3 loop with little evidence of antibody development (Fig. 23). Our findings apparently highlight the impact of a single amino acid mutation in the V3 region to selectively promote either a TH1 (CMI) or TH2 (antibody) response.

Enhanced induction of TH1 responses, IL-2 and IFN- γ cytokine production by D-UG23c. The immunogenic potential of D-UG23c to elicit CMI was further analyzed in cytokine synthesis assay. Splenocytes were isolated from mice previously primed with the ENV plasmids and culture supernatants of these cells were tested for the secretion of IL-2. Immunization with D-UG23c stimulated IL-2 levels of at least twice that recorded for the mutant clone, mD-UG23c ($n=3$, $p < 0.017$) or the control vector, CMVint-BL ($n=3$, $p < 0.0026$) (Fig. 24). Further, data derived from an intracellular cytokine assay demonstrated that the enhanced IL-2 accumulation was restricted to CD4 rather than CD8 cells of mice immunized with D-UG23c (Fig. 25). The CD4 and CD8 cells recovered from these mice secreted greater amounts of IL-2 ($n=5$, $p < 0.002$) and IFN- γ ($n=5$, $p < 0.0006$) respectively, as compared to cells from mice inoculated with the control vector, CMVint-BL. Consistent with the results of the cytokine secretion assay, the D-UG23c immunogen was also more potent in the promotion of IFN- γ synthesis as compared to the single point mutated V3 loop of mD-UG23c ($n=5$, $p < 0.008$) or the B cell immunogenic clone, C-UG045 ($n=5$, $p < 0.009$) for CD8 cells. Collectively, these findings are consistent with our hypothesis that clones that express GPGX in the V3 region selectively

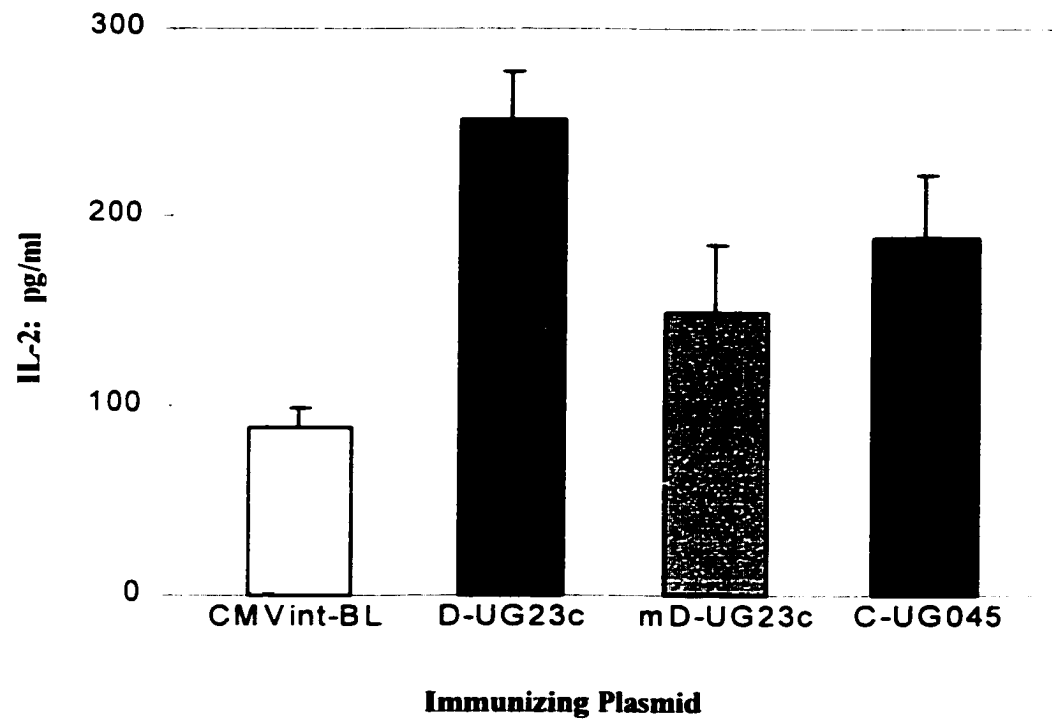
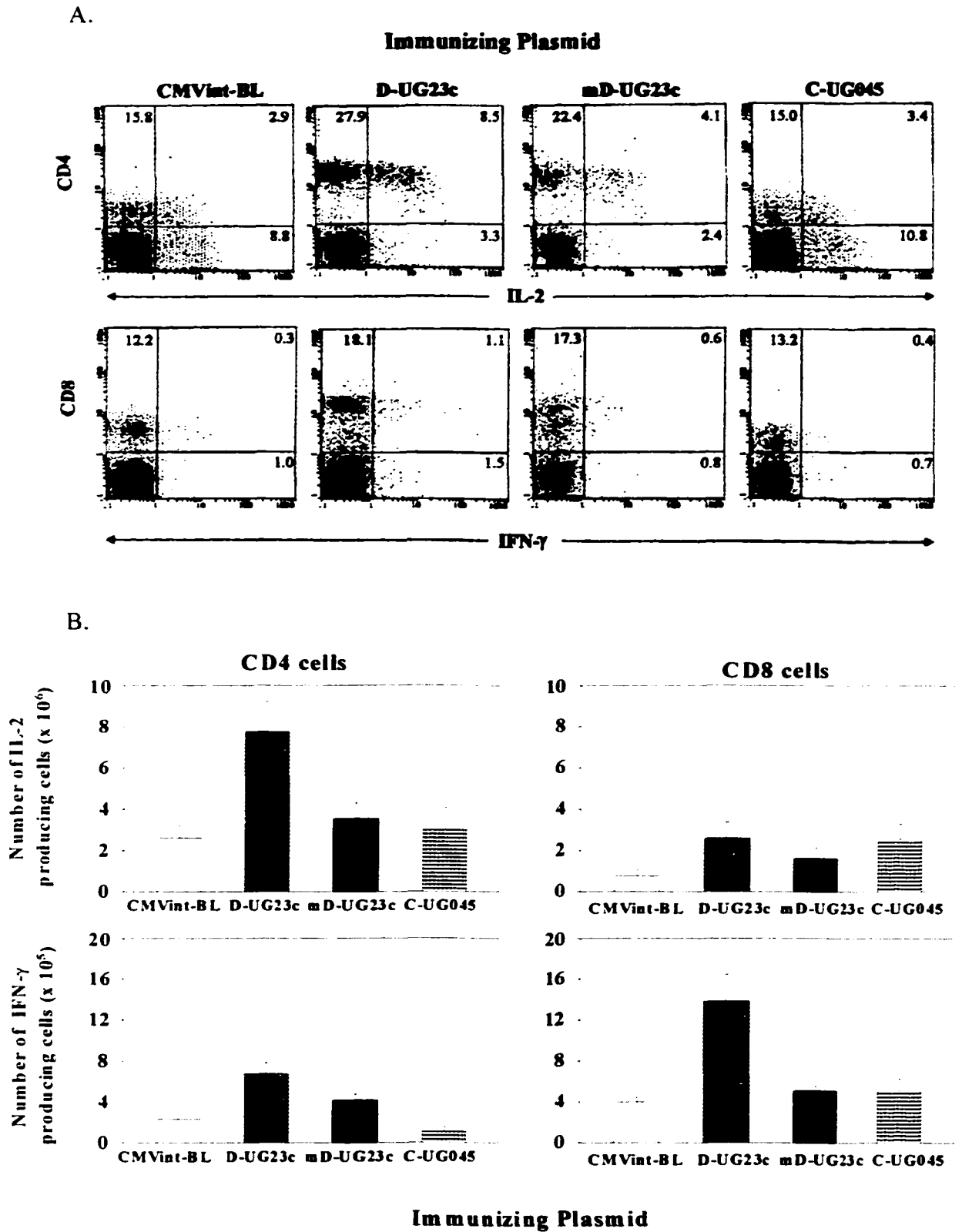


Figure 24. IL-2 secretion by cultured cells from mice immunized with control plasmid vector: CMVint-BL or plasmids encoding D-UG23c, mD-UG23c or C-UG045. Data shown are the mean and standard deviations (SD) of data from 3 individual mice (different shaded bars) in each immunization protocol. Background values for IL-2 secretion from non-immunized cells (75 pg/ml) were subtracted from the data shown.

Figure 25. IL-2 and IFN- γ accumulation in T cell subsets after ENV encoding plasmid immunization. (A) Representative FACS profiles of IL-2 and IFN- γ accumulation in electronically gated CD4 or CD8 cell subset. The percentage of cytokine secreting cells is shown in each panel. (B) Relative numbers recovered from splenocytes of IL-2 and IFN- γ expressing cells post immunization. Non-immunized mice showed the same profile as those immunized with CMVint-BL ($p > 0.5$). The data shown were generated after analyses of the results in (A). Cell numbers were determined after FACS analysis using electronic gating as described in Pestano et al. (52). Each value represents the mean of cytokine accumulation for mouse analyzed. Error bar (SD) is shown for groups of 5 mice tested.

Figure 25



elicit humoral responses while down-regulating TH1 responses. Further, the levels of IL-2 or IFN- γ induced by C-UG045 were not significantly different from that elicited by immunization with the GPGQ reconstituted mutant, mD-UG23c (Fig. 25).

Suppressive effect by immunogen encoded by D-UG23c to antibody development. Taken together these data raised the possibility that TH1 response by D-UG23c may also affect antibody generation by β -turn competent gp120 clone. The antibody reactivity showed that immunization with D-UG23c clone for 3 times followed by priming with C-UG045 did not allow elicit an antibody response (Fig. 26). Interestingly, the reverse experiment showed that D-UG23c immunogen play an important role for down regulation of antibody production from mice immunized with C-UG045 (Fig. 26). Since immunization with D-UG23c apparently inhibit antibody generation. These results suggest that an inverse relationship exists between TH1 and TH2 epitope in the control of immune reactivity to gp120.

Figure 26. Suppressive effect of D-UG23c on antibody response. Each line represents the ELISA reactivity of anti-V3 loop antibodies induced by immunization with the indicated plasmid. Mice were immunized according to the following sequence (A) C-UG045 followed at 8-20 weeks by C-UG045 (◆), D-UG23c (■), C-UG045 + D-UG23c (▲) or CMVint BL (●) (B) D-UG23c followed by C-UG045 (◆), D-UG23c (■), C-UG045 + D-UG23c (▲) or CMVint BL (●) (C) C-UG045 + D-UG23c (◆), C-UG045 + CMVint BL (■), or D-UG23c + CMVint BL (▲). C-UG045 V3 peptide was tested in ELISA. Error bars (SD) shown are for group of 4 mice tested at each time point. The level of V3 loop antibodies to D-UG23c was same as background value (data not shown).

Figure 26

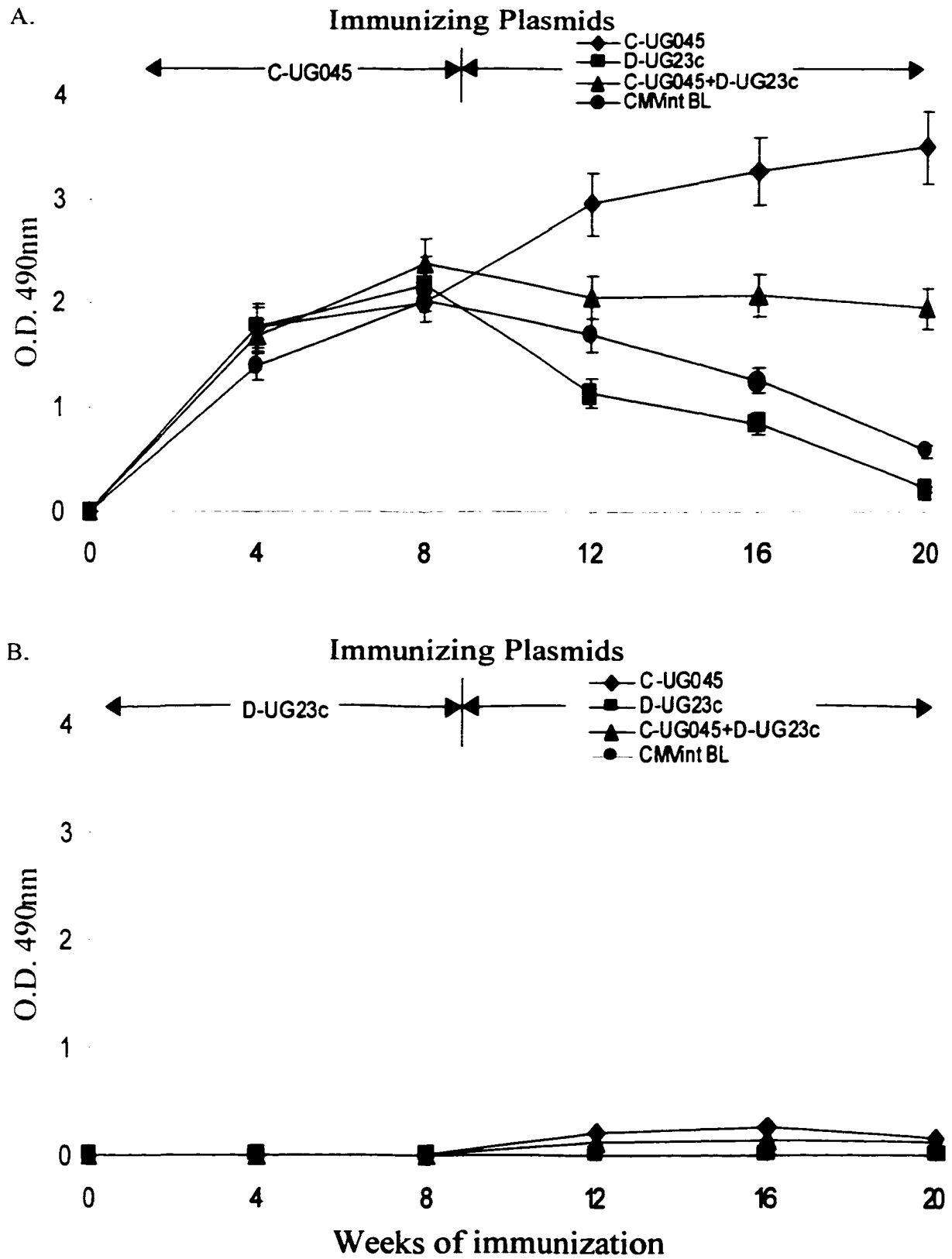
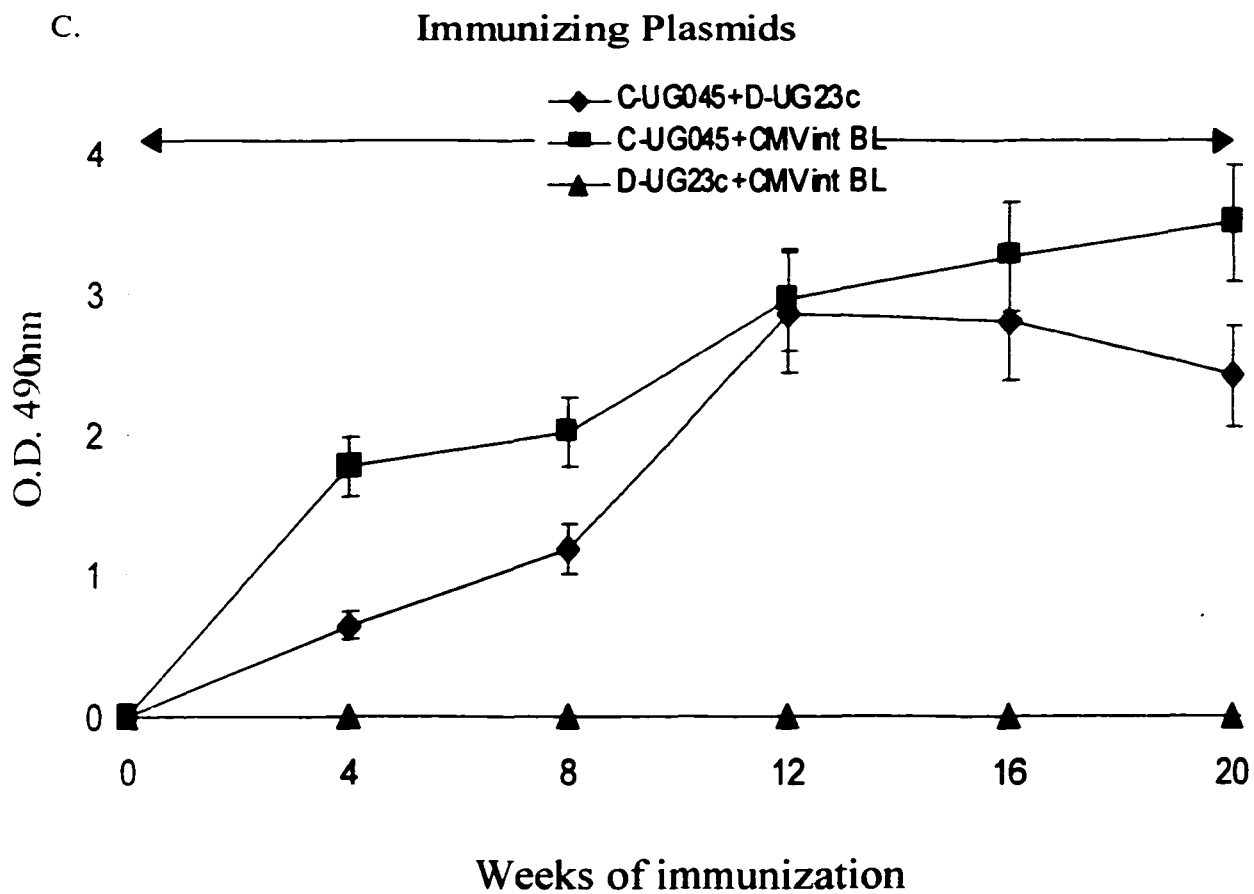


Figure 26



DISCUSSION

The experiments described in this report have analyzed HIV-1 field isolates that encode gp120 molecules which express conformationally distinct variants of the V3 loop. One subset of the apical V3 sequences encoding the GPGX residues, was predicted to have a high likelihood of adopting a β -turn conformation and elicited vigorous antibody responses in DNA-mediated immunization. In contrast, I observed that V3 loop variants with the apical residues GLGQ and GQGQ encoded by the clones D-UG23c and D-UG042, respectively, failed to generate antibody response. The presence of GLGQ or GQGQ at the cap of the V3 loop expressed by these clones was apparently associated with the reduced ability to stimulate significant antibody production (Fig. 15). This effect was hypothesized to be due to the loss of the antigenic β -turn structure in the predominant neutralizing epitope in V3 loop of gp120 (Fig. 17). In keeping with this proposal, the replacement of GLGQ with GPGQ in D-UG23c using site-directed mutagenesis yielded rgp120 molecules that promote significant antibody production directed at the V3 loop (Fig. 21). Substitution of the hydrophobic leucine with a proline residue possibly enhances surface accessibility of the apical loop residues for B cells (35, 45). The ability to suppress or enhance the relative immunogenicity of a selected epitope in gp120 using mutagenesis assays bear significant implications for vaccine research and development.

Point substitutions that alter the encoded V3 loop have been shown to confer resistance to antibody-mediated neutralization of HIV-1 (59). The molecular mechanisms which underlie the remarkable phenotypic effects of seemingly minor mutations are

unclear. LaRosa et al. (42) suggested that selective pressure may favor the conservation of a β -strand- β -turn- β -strand conformation at the cap of the V3 loop in HIV-1 gp120 molecules. Indeed >75% of field isolates thus far analyzed tend to express the conserved tetrapeptide GPGR or GPGQ tetrapeptide despite extensive variation in the V3 loop (21). Our findings indicate that both the GPGR and GPGQ peptides confer a high probability of a β -turn conformation occurring at the cap of the loop (Fig. 17) as previously reported (35, 36, 45). In contrast, the β -turn deficient apical tetrapeptide GLGQ encoded by D-UG23c, a clone analyzed herein, (Fig. 15) is observed in \cong 5% of the clinically recovered isolates (21). X-ray crystallographic analysis have also indicated that broadly neutralizing monoclonal antibodies also bind to the β -turn region (GPGR) of the loop (60). Our results suggest that the loss of the β -turn structure significantly alters the immunogenic potential of the V3 loop. This change can be effected by the mutation of a single critical amino acid residue in the loop. I have apparently identified a novel molecular mechanism that may allow HIV-1 to evade humoral immune responses through the alteration of a minimal number of amino acids that may be vital for the exposure and antibody recognition of the V3 loop.

Although unable to generate high titers of V3 loop antibodies, immunization of mice with the β -turn deficient clone D-UG23c was shown to promote a strong CMI response when tested in a proliferation assay (Fig. 23). Further, splenocytes from mice immunized with D-UG23c were enriched in CD4 and CD8 cells that secrete IL-2 and INF- γ as determined by FACS analysis (Fig. 25). This observation raises the possibility that D-UG23c and its mutated clone mD-UG23c may be useful reagents for the selective induction of CMI or antibody response, respectively, at least in experimental vaccination.

The arm of the immune response that may well be enhanced by immunization with D-UG23c is apparently TH1 driven. Previous studies have suggested that IL-2 and other TH1 responses contribute to the control of a primary infection and AIDS pathogenesis (61-65). HIV-1-specific CTL reactivity is reported to precede antibody development in a primary infection and has been associated with the control of the initial burst of viremia (61, 62). A strong CTL response apparently accounts for the slow progression to AIDS in some HIV-1 seropositive individuals (63-65). The frequency of the virus-specific CTL subsequently decreases along with a concomitant rise in viral replication and the onset of AIDS (61-64). Based on this scenario of a TH1 function several attempts have been made to modify or eliminate the V3 loop with the aim of suppressing the overwhelming antibody response to the V3 (55, 66, 67). The underlying rationale in some of these studies is that a strong TH2 and the associated antibody responses to the V3 may counter-regulate and occur at the expense of a TH1 type reactivity. An inverse relationship has been proposed to exist between TH1 and TH2 reactivity based on prospective studies of immune response in HIV-1 infection (28) and in planned immunization with gp160 in mice (68). The basis for the TH1 → TH2 switch during the infection or repeated immunization is not clearly understood, but it may lie in the regulatory interaction that occurs between these distinct arms of the immune response. Support for this view comes, in part, from studies showing that the addition of IL-10 or IL-4 to cells in culture down-regulates CTL reactivity to HIV-1 antigens (69). In view of these observations a strong justification exists for the development of HIV-1 immunogens that would induce and sustain TH1 immune responses, CTL or cytokine production to support vaccine development.

Indeed, an inverse relationship was subsequently described between HIV-1 specific CTL reactivity and viral load in long-term survivors (69). The finding that a naturally occurring variant of gp120 may maximize CMI response in a selective manner appears to offer the opportunity to assess the relative contribution of the different facets of immune response in the neutralization of HIV-1.

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