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IMMUNOLOGICAL CHARACTERIZATION OF gp120 FROM NEW YORK HIV-1 SUBTYPE B ISOLATES.

By

JANICE RILEY

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

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Abstract

IMMUNOLOGICAL CHARACTERIZATION OF gp120 FROM NEW YORK HIV-1 SUBTYPE B ISOLATES.

by

Janice Patricia Riley

Adviser: Dr. William Boto

Human immunodeficiency virus type 1 (HIV-1) has undergone remarkable genetic divergence over time. The accumulation of mutations is particularly pronounced for the envelope (ENV) gene. This region of the provirus encodes the leading subunit candidate vaccine, gp120. The dynamics of transmission and distribution of divergent strains of the virus are important issues in the current search for promising immunoprophylactic therapies to control the AIDS epidemic. To determine the impact of genetic diversity on the immunogenic properties of gp120 encoded in North American subtype of HIV-1 a 625 bp (C2 to V5) fragment of ENV was amplified and sequenced.

Phylogenetic tree analysis of the nucleotide sequences showed that the ENV clones B_RT1.4, B_RT1. 15, B_RT1. 17, B_RT1. 21, derived from the patient isolate RT1 and the ENV clones B_RT3.6, B_RT3. 10, B_RT3. 11, B_RT3.12 and B_RT3.15 from the isolate RT3, cluster with the "subtype B" viruses. Marked intra-patient and inter-patient sequence variation was recorded over the region analyzed. To assess the

effect of the primary sequence variation on the distribution of linear antigenic epitopes, the complementary software programs SURFACE PLOT and PEPTIDE STRUCTURE were used to analyze the region. Eight analogous but distinct antigenic sites designated E1 through E8 were identified in gp120 derived from RT1 and RT3 despite the marked divergence in the primary (nucleotide and amino acids) sequences. Synthetic peptide comprising the E2[V3] epitope in RT1 and RT3 displayed varying ELISA reactivities with sera from asymptomatic HIV-1 infected New York donors.

To further determine the immunogenic potential of gp120 encoded in the sibling clones of RT3 and RT1 immunizations were conducted with naked ENV plasmids and with V3 loop multiple antigenic peptides (MAPs) from each isolate. Inoculation with the ENV clones and MAPs from the RT3 isolate elicited antibodies that reacted highly with homologous and heterologous PND peptides in ELISA. In a sharp contrast, inoculation with the ENV plasmid and MAPs from the RT1 derived clones elicited near background level of antibody responses. Further, V3 derived MAPs and DNA-mediated immunization with ENV clones of RT3 generated superior cell-mediated immune response compared to immunization with the RT1 derived clones.

These findings demonstrate a marked impact of sequence variation on the immunogenetic properties of gp120 encoded in these HIV-1 field isolates, and could complement the current search for globally effective vaccine candidates.

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This work is dedicated to the loving memory of my brother, Junior Anthony Riley.

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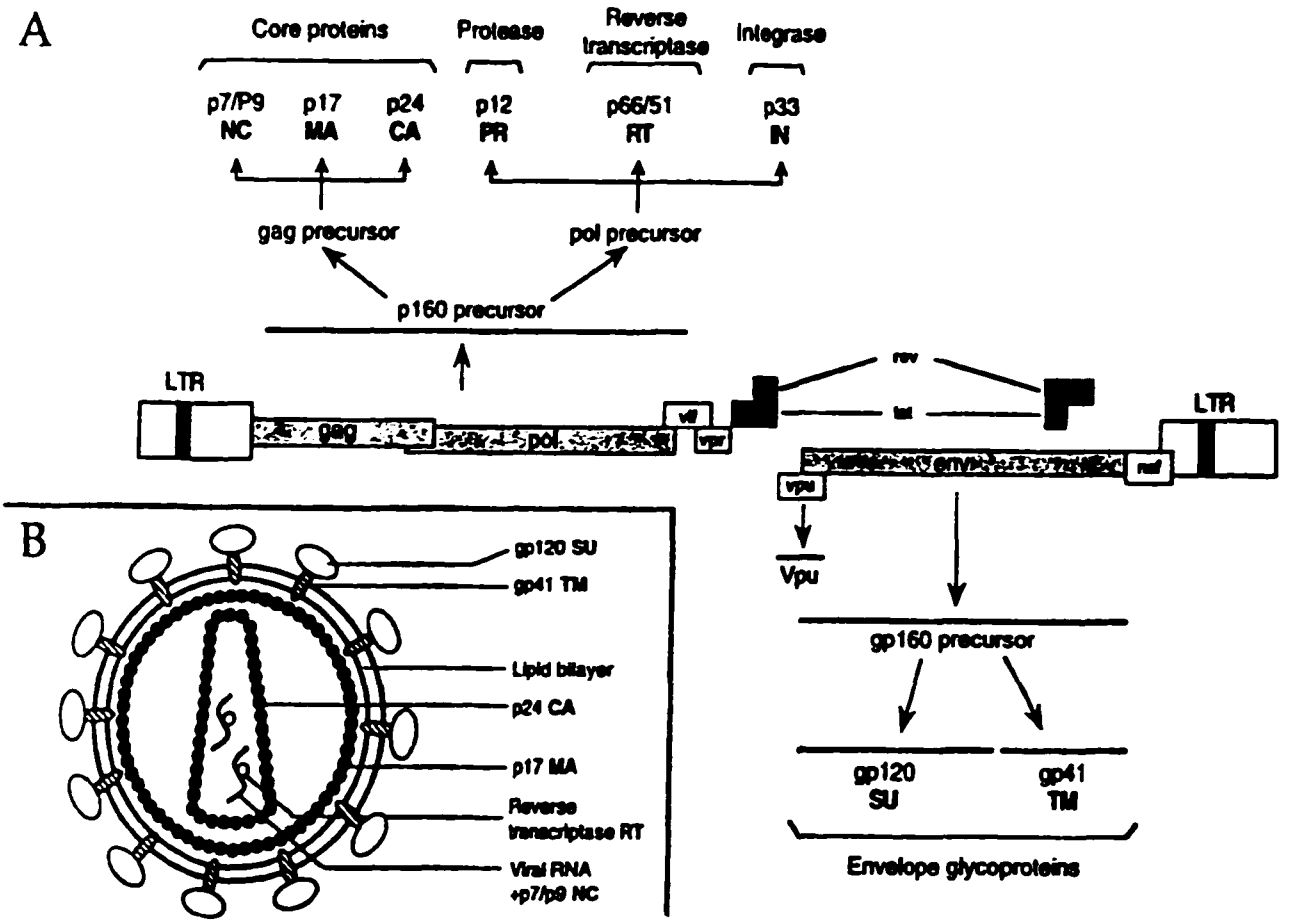
INTRODUCTION

Intravenous high-drug users are at a greater risk of exposure to HIV-1 than most other subsets of the society (1). HIV-1 infection has been reported among injecting drug users (IVDUs) in over 95 countries (1-7). According to findings from the Centers for Disease Control (CDC), drug users currently account for an estimated 40% of all acquired immunodeficiency syndrome (AIDS) cases reported in the USA (8). Since the epidemic began, injection drug use has directly and indirectly accounted for more than one-third (36%) of AIDS cases in the United States. This disturbing trend is on the rise. Of the 48,269 new cases of AIDS reported in 1998, 15,024 (31%) were IDU-associated.

The increased risk of IVDUs contracting the virus is clearly related to the higher numbers of sex partners and the sharing of contaminated injection paraphernalia that occur within this group (2-8). More than 60% of IVDUs currently enrolled in a methadone maintenance program at Addiction Research Treatment Center (ARTC) in New York City are HIV-1 seropositive (Brown, L., personal communication). Other findings (1-9) also support the view that drug addicts constitute a high-risk target population most likely to benefit from the availability of an effective immunoprophylatic therapy.

One of the more promising strategies to combat the escalation of the AIDS epidemic within the community of IV drug users and worldwide is the development of an effective antiviral vaccine (10). However, the complexity of life cycle of HIV-1 has presented major obstacles in the development of vaccine (11). Infection with the virus involves multiple steps, involving several unique viral regulatory and structural proteins (Figure 1) (11, 12).



Key:

SU; surface

TM; transmembrane

CA; capsid

MA; matrix

Figure 1. Schematic of HIV-1 proviral genome and the viral particle. (A): genetic map of HIV-1 proviral DNA showing the expression of regulatory and structural proteins. (B): a cross sectional representation of the mature virion (adapted from reference 19).

The surface glycoprotein gp120 (Figure 1B) is responsible for mediating virus attachment to the CD4 molecule in the initial stage of infection (Figure 2) (11-14). HIV-1 destroys $CD4^+$ cells that it infects by two forms of cytopathic effects; syncytium formation and single cell lysis (11). Thus HIV-1 is tropic for the $CD4^+$ T cells and macrophages due to the high affinity interaction between gp120 and the CD4 molecule. The fusion of the cellular membrane and the envelope glycoprotein subunits (11, 14-21) appears to facilitate viral entry. Although CD4 binds to gp120, the expression of CD4 is not sufficient to allow for the infection of nonhuman cells and some CD4 positive cells (21-23). Several cofactor (s) that may function in conjunction with CD4 have been postulated (21). It has now been shown that the fusion and entry of HIV-1 is mediated by G-protein coupled chemokine co-receptors (CXCR4, CCR5 [CCKR5], CCR3, CCR2, CCR8) found on the host cell surface (24-34) (Figure 3). The glycolipid galactoryl ceramide (GalC) has been shown to mediate infection in both neural and epithelial cell lines (35, 36). Upon penetration of permissive cells, the HIV-1 nucleocapsid is uncoated and the genomic RNA is reverse-transcribed. The proviral DNA is then circularized and duplicated (Figure 2). The duplex DNA molecule is transported into the nucleus where it is integrated into the host chromosomal DNA (Figure 2). The subsequent activation of the infected lymphocyte by antigen (or other agent) triggers the initial transcription of the host and viral mRNAs (reviewed in 11). During replication two classes of viral transcripts are synthesized: one class remains in the nucleus and is processed by a series of complex splicing reactions to provide the regulatory and structural proteins; the other class is transported to the cytoplasm without processing to serve as the genomic RNA for new emerging virions (Figure 2). The factors and mechanisms that select the sites for chromosomal integration and the replication cycle of the virus are the current targets of research for chemotherapeutic interventions (11, 37, 40).

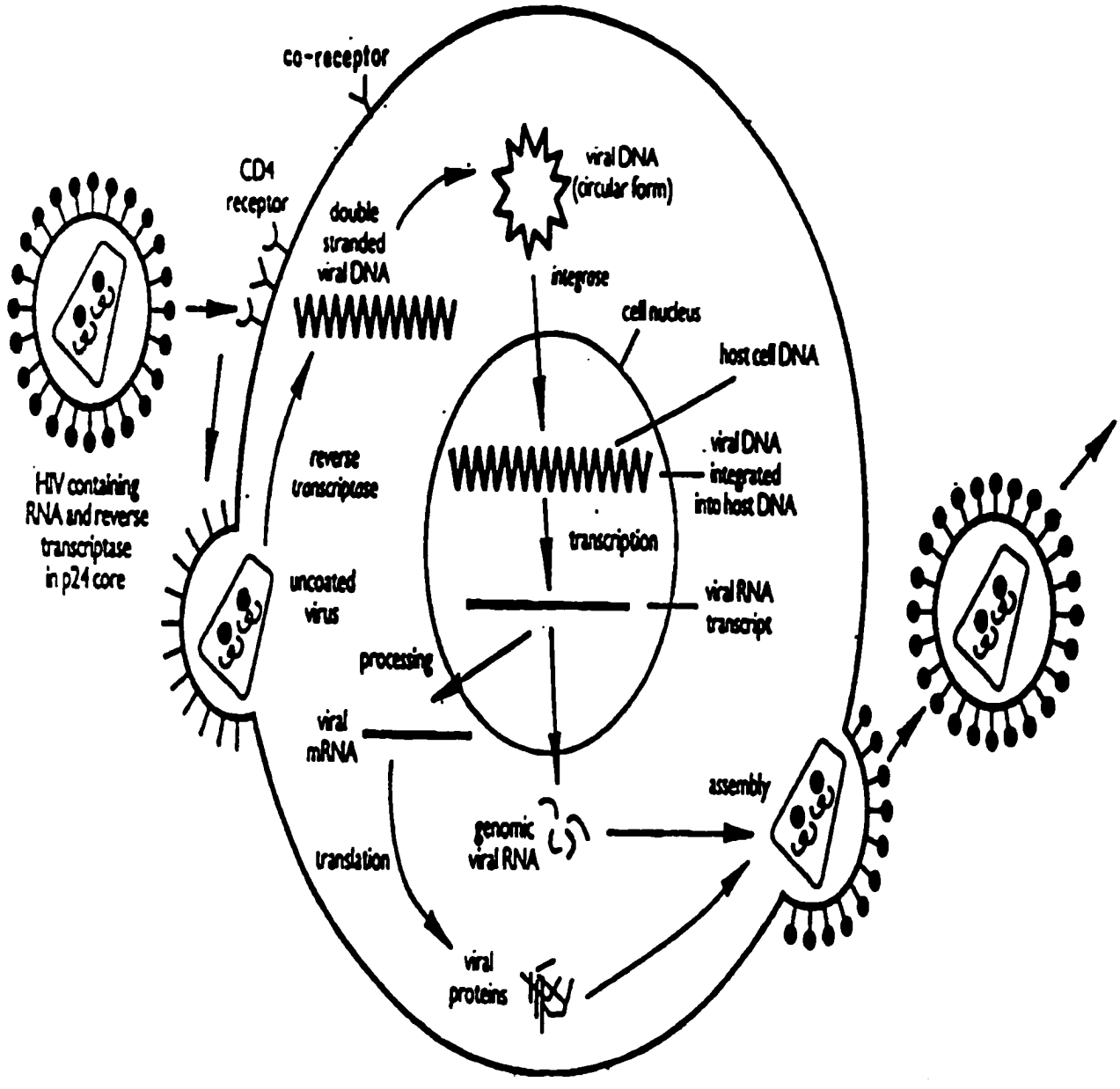


Figure 2. Schematic representation of the replication cycle of HIV-1 (Adapted from reference 37).

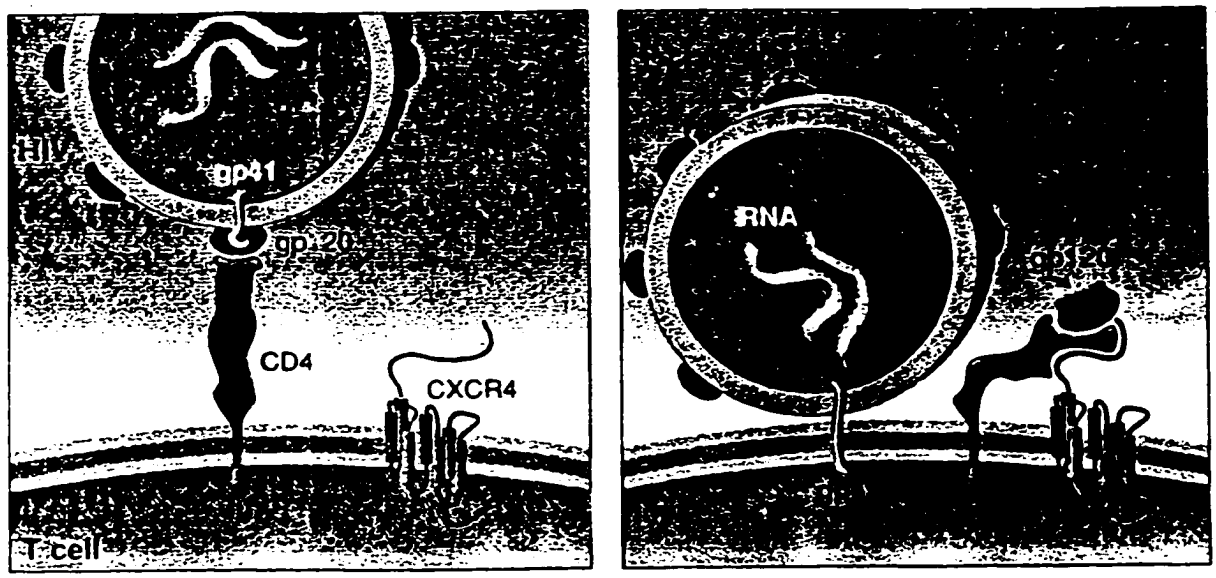


Figure 3. Schematic representation of how HIV-1 uses chemokine co-receptors like CXCR4 to enter T cells (adapted from reference 38).

Current chemotherapy for HIV-1 is limited to four reverse transcriptase inhibitors (zidovudine [AZT], didanosine, zalcitabine, and stavudine). However, the clinical efficacy of these inhibitors has been limited by inadequate viral suppression, the emergence of resistant viral variants and adverse side effects (41). HIV aspartyl protease is a virus-encoded enzyme that is essential for the processing of GAG and GAG-Pol precursors into structural proteins required for virion production (42). A number of potent HIV-1 protease inhibitors including saquinavir, indinavir, ritanivar have been described (43-45). Potent

combinations of these antiviral agents have been developed that can significantly inhibit viral replication and spread (46-48). Administration of these inhibitors can also reduce viral RNA levels (44, 49, 50). Despite effective suppression of viral RNA in most individuals receiving triple therapy, integrated proviral DNA can be detected for prolonged periods of time in a variety of cells and in the lymphoid tissues (51, 52). Further, similar to results obtained with reverse transcriptase inhibitors (41) viral variants with reduced sensitivity to these inhibitors have emerged in cell culture and in patients (46, 47, 51). In addition, these antiviral therapies require strict compliance (52) and are very costly. Therefore, a vaccine that confers universal protection from HIV-1 appears to hold the greatest promise.

Efforts to develop HIV-1 candidate vaccines have focused on the external envelope glycoprotein, gp120 (Figure 4) (11, 18, 53, 54). In HIV-1 infected individuals, three types of neutralizing antibodies have been described: (i) those that bind to the linear epitopes in the V2 or V3 region of gp120 (56-58); (ii) those that recognize conformational epitopes that overlap the CD4 binding site (15, 42, 59, 60) and (iii) those that bind the epitopes in the transmembrane glycoprotein (gp41) (61). However, several planned immunization and challenge experiments in different animal models have shown that gp120 is the major target for the immune response that neutralizes HIV-1. A considerable fraction of virus neutralizing antibodies are directed against this glycoprotein (52, 53, 55, 62-67). Recently, the crystal structure that depicts interaction between gp120, core CD4 receptor and a neutralizing antibody was described (68). These findings strengthen the notion that gp120 might be a useful component of a subunit vaccine. However, data from several previous studies have raised concerns for vaccine researchers: (a) some of the chronically infected cells such as macrophages and glial cells do not express viral antigens, and may serve as reservoirs (69) for the expansion of the viral pool in vivo (reviewed in 11, 69, 70); (b) the integrated HIV proviral DNA may be inaccessible to any of the known immune responses until cell lysis (reviewed in 11);

(c) transmission may be accomplished through multiple mucosal surfaces, and distinct mechanisms of the viral life cycle may require different modes of immune intervention, (reviewed in 11, 67); (d) tissue-specific strains of HIV-1 have been identified (71, 72); (e) during the period of "clinical" latency, active viral replication can be detected in the lymphoid organs (73) and ~10^{10} virions are produced each day (74, 75); (f) infection involves free or cell-associated virus particles (11) and (g) high genetic variability exists in the viral genome and could consequently lead to antigenic variation (10, 11, 76). These factors are important to consider in the design of the next generation of HIV-1 candidate vaccines.

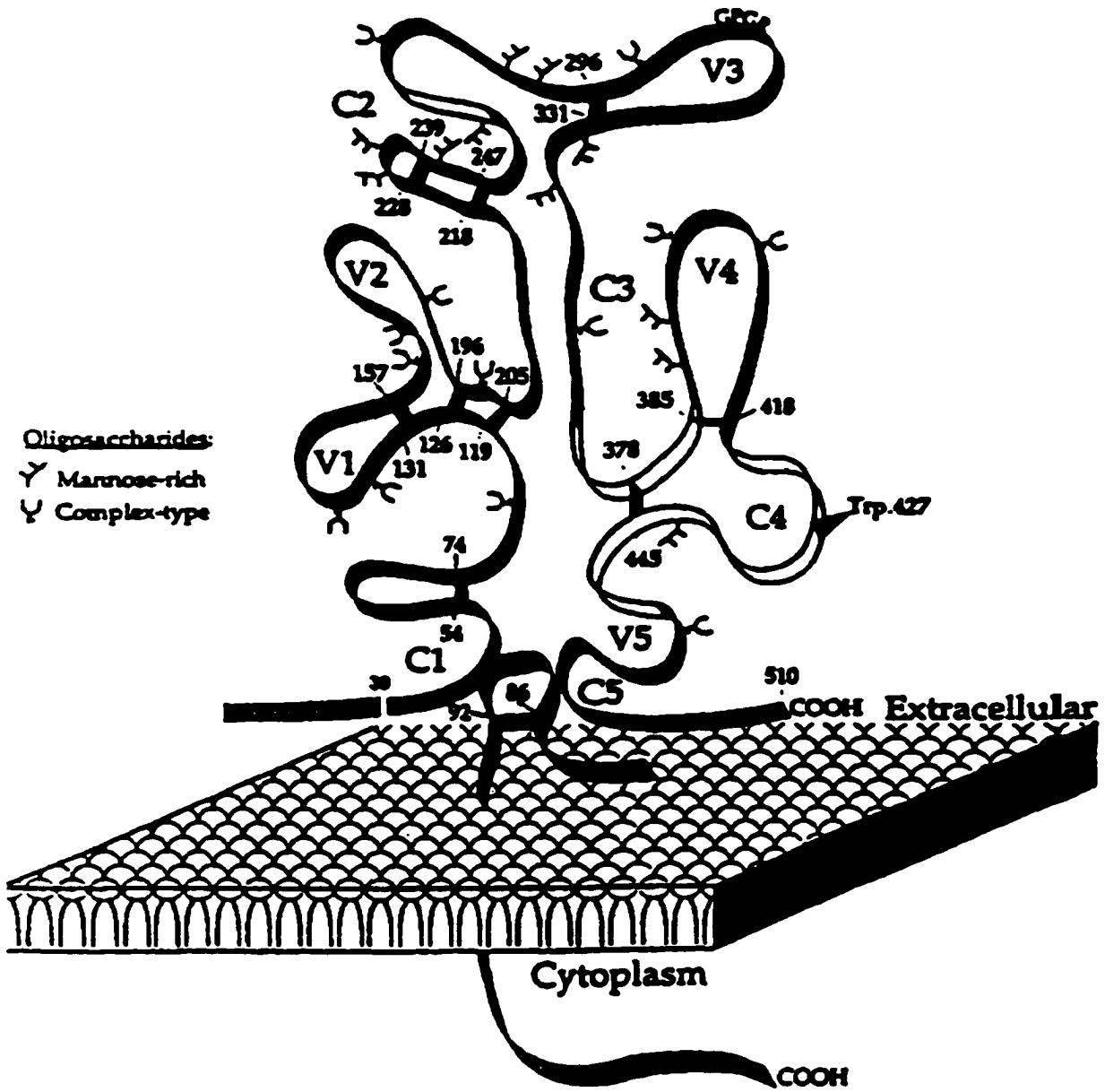


Figure 4. Schematic representation of a leading candidate immunogen, gp120. The constant regions, C1 to C5, and the variable regions, V1 to V5, are shown (adapted from reference 37).

Another obstacle to vaccine research is the evidence for multiple cellular targets that exist for HIV-1 (10, 69, 71, 76). The ability of the virus to infect different types of cells varies from isolate to isolate and is referred to as cellular tropism. HIV-1 isolates may be classified as macrophage tropic (M-tropic) or T cell tropic (T-tropic) (77-79).

M-tropic non-syncytium inducing (NSI) isolates infect primary macrophages but fail to infect transformed T cell lines (79, 80). In contrast, T-tropic syncytium inducing (SI) strains grow in transformed T cell lines (80). NSI viruses appear to be preferentially transmitted by sexual contact and constitute the vast majority of versions found in a newly infected individual (80). The SI virus generally appears late in the course of the infection during the phenotypic switch that often precedes the onset of clinical symptoms (AIDS) (81, 82). The viral determinant of cellular tropism has been mapped to gp120, in particular to the third variable region (V3 loop) (82-85). Studies to delineate the basis of cellular tropism have led to the identification of some of the co-receptors for HIV-1 (24, 25). Feng et al. (24) have reported that CXCR4 is the co-receptor for efficient entry of T-tropic strains of HIV-1 into target cells. Viruses using CXCR4 are termed "X4" (32). In addition, antibodies raised against CXCR4 and some β–chemokines (RANTES, MIP-α, MIP-β) blocked the entry of T-tropic but not M-tropic strains into target cells. Several reports have shown that the β chemokine receptor, CCKR5 (CCR5) is the co-receptor for M-tropic HIV-1 strains (24-34). Viruses using CCR5 are termed "R5" (32). As research in the field progressed at a fast pace, it is now known that HIV-1 use a variety of chemokine receptors more recently extended to include CCR-2b, CCR-3, CCR-8 (24-34) as well as some orphan receptors (STRL33/BRONZO, BOB/GPR15) (34, 88, 89). Several studies have shown that a variety of primary isolates (dual –tropic viruses) have the potential to use more than one co-receptor (90, 91).

The proposed role of CCKR5 (CCR5) is supported by several other reports. In a large cohort (1,955 individuals) it was shown that the homozygous deletion (32 bp) of CCKR5 (CCR5) strongly associates with resistance to HIV-1 in high-risk individuals (92, 93) and the heterozygotic allele strongly associates with slow progression to AIDS (long-term survivors) (92). To date, evidence for the protective mutation has been found primarily in Caucasians but not in Asians and Africans (92-94). Studies of HIV-1 infected cohorts have revealed no homozygotes among the latter group (94-97). The new findings concerning the cellular co-receptors for HIV-1 and the cellular tropism of the different strains of the virus could advance an understanding of viral-induced pathogenesis and may suggest new therapeutic strategies to control the AIDS epidemic.

The marked genetic and antigenic variation of HIV-1 found worldwide is another major obstacle for current vaccine research efforts (96). The source of variation is the infidelity of the viral reverse transcriptase which lacks exonuclease activity (11, 77). This variation is manifested at several levels: within the individual, HIV-1 exists as multiple substrains of closely related viruses (quasispecies) (98-102). The number of intra-patient variants increases as the disease progresses (reviewed in 11). There is also variation between isolates obtained from different individuals infected with the virus in the some geographical area (96). Further, isolates identified in distinct areas of the world display a high degree of genetic divergence (96). Intersubtype recombination is an important additional source of HIV-1 genetic variation. Analyses of full-length genome sequences have revealed the inter-clade recombinants, A/C, A/D, ADI, and G/H (103-105). Variation in HIV-1 could influence viral pathogenesis by generating viral strains that differ in virulence, which may in turn affect the pace and severity of disease (77, 79). Escape mutants that resist specific humoral and CMI neutralization may also arise (106-108). The escape mutants may arise during the replication cycle of the virus as well as under selective pressure from the immune response of the host (77, 79). During the natural course of infection, high and sustained anti-gp120

antibody levels are present, which neutralize the cell free virions (106). However, during the progression to AIDS the immune system may become overwhelmed by the viral load and it eventually collapses (10, 77, 79). In addition, the current working hypothesis of HIV-1 infection proposes that a high CD4 cell production and turnover is the result of T cell destruction (10 billion CD4 cells are infected and destroyed daily). The long-term outcome of this massive production/destruction of cells is the exhaustion of the immune system (47). However, work recently reported by Hanley et al. (109), together with a series of other studies have demonstrated that the decrease in CD4 cell count is caused by a shortened survival time and a failure to increase T cell production (110-112). The typical course of HIV-1 infection is illustrated together with the kinetics of neutralizing antibody and cytotoxic T cell (CTL responses (Figure 5). However, it has become clear that the course of the disease can vary widely (109).

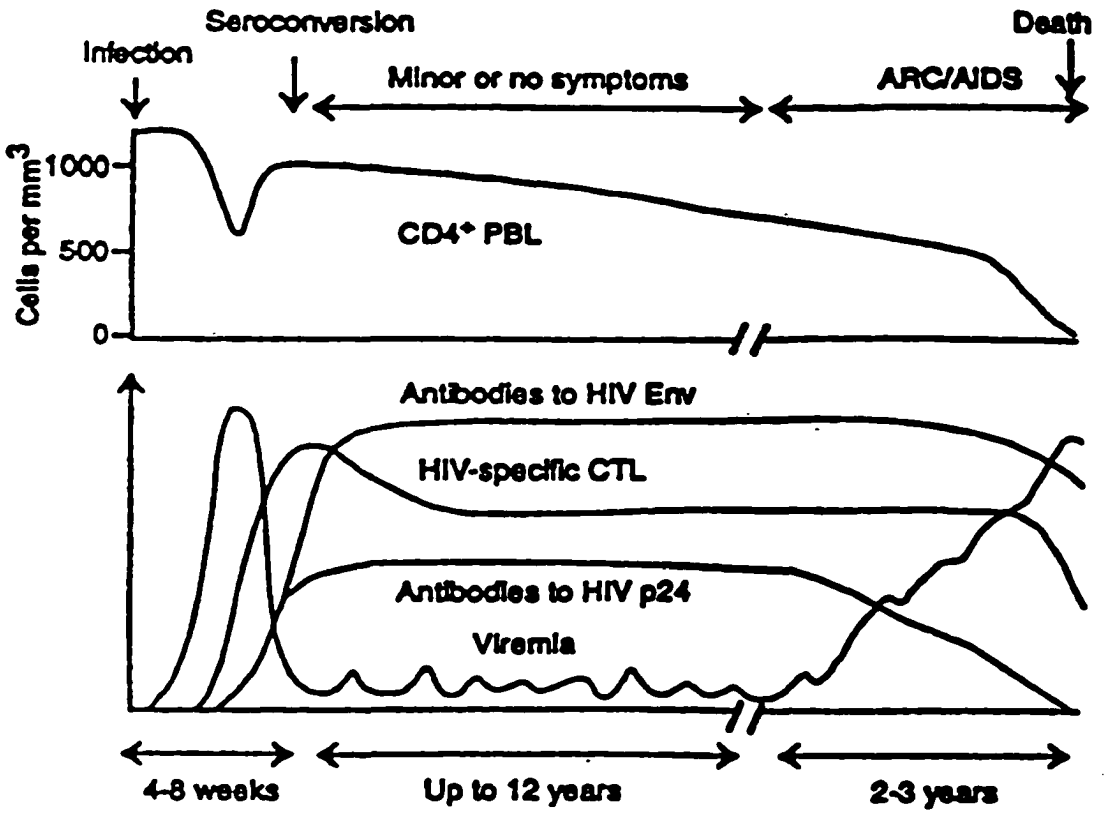


Figure 5. Schematic course of HIV-1 infection showing the relationship of $CD4^+$ cells and disease progression (40).

Phylogenetic analysis of the nucleotide sequences of the envelope (ENV) and core (GAG) genes of numerous HIV-1 virus strains has resulted in their classification within a major (M) group, which is subdivided into 10 genetic subtypes, and a genetically distant

outlier (O) group. Different subtypes are unevenly distributed in different parts of the world (97, 113-115). In all, the AIDS virus has been shown to cluster into at least twelve major phylogenetic subtypes or clades: the M group consisting of subtypes A-J; the outlier group (O) and the recently described subtype N based on the nucleotide sequence analyses of the ENV and GAG sequences (96, 113-116). Five subtypes (A, B, C, D, and E) show over 20% divergence over a 300 bp region of ENV (97). Nearly all the HIV-1 vaccine candidates developed to date have been based on the laboratory strains of the clade B virus (11), the prevalent subtype in America, Europe and Australia. However, the most rapid spread of the HIV-1 virus is in the developing countries where subtype C accounts for 20%-30% of the infection. This observation underscores the importance of the ongoing studies to characterize isolates from defined high-risk cohorts in support of broadly effective candidate vaccines (116-130). Although several subtypes coexist in many parts of the world, some geographical regions apparently harbor predominant subtypes. The subtypes A, C, and D are common in sub-Saharan Africa (97, 119-122); whereas nearly all of the viruses identified in North and South America, the Caribbean and Europe are of the B group (97, 123). The subtypes B, C and E viruses are highly prevalent in India and Thailand (99, 124, 130), while subtypes B and F are the dominant subtype thus far reported in Brazil (97, 125) and Romania (99, 125). Strains from the subtypes G, H, I, N and O have been identified in West Africa (114, 115, 127, 128). This genetic variability is perceived by many as a major obstacle to the development of a broadly effective or "global" HIV vaccine.

Several ENV clones from diverse geographic areas that are yet unclassified have been assigned the "U" subtype (97). The global spread of HIV-1 has been linked to the migration of individuals as a result of natural disease and man-made conditions (e.g., civil war, drug

trade and economics). Such migration may subsequently lead to dispersion of diverse HIV-1 subtypes into regions previously affected by a more genetically restricted virus. This eventuality should be considered in formulating strategies for the global control of the HIV-1 pandemic. At present, the prospects for the development of a universally efficacious vaccine(s) which elicits protective immunity seem remote. However, protective rather than infection preventing vaccines may provide relief from the epidemic (131).

The goal of vaccination is to induce immunity that protects the host from disease. Candidate vaccines should be able to generate long-term protective immune responses against specific antigens. A controversial issue in the analysis of immunity to HIV is the need for a sustained protective immune response to the virus. Some investigators have expressed doubt that the development of an effective vaccine is feasible (132, 133). Much of the disagreement stems from a lack of understanding of the correlates of protective immunity to infection and also the lack of a consensus of an appropriate animal model that could be used to establish evidence of protection. However, some evidence in support of protective immunity has emerged from immunization experiments conducted in animal models, and also from analyses of the natural history of human infection. It has been shown that partial to complete protection can be achieved in macaques when challenged with the homologous Simian Immunodeficiency Virus (SIV) after immunization with various adjuvant formulations of inactivated SIV. In a study reported by Hue et al. (134), it was found that immunization with soluble recombinant gp120 of SIV could protect against a low dose challenge with the homologous virus. Similar studies have indicated that antibodies raised against recombinant gp120 can protect chimpanzees against challenge with HIV-1 (135, 136). Studies using live recombinant canary pox virus expressing gp120 derived from MN or various combinations of HIV-1 antigens indicated a protective effect for chimpanzees subsequently challenged with heterologous HIV-1 (136). Additionally, Neurath et al. (64) have shown that immunization of chimpanzees with gp120 followed by boosting with V3

peptides can elicit high titers of neutralizing antibodies. These studies argue for the potential efficacy of a gp120 subunit immunogen, especially when employed in a combination with other vaccine strategies. In addition, several studies have further shown that rhesus monkeys vaccinated with a nef-deficient, live SIV were completely protected from intravenous challenge with a fully virulent strain (137, 138). However, safety concerns have been expressed that an attenuated mutant of HIV-1 similar to the nef-deleted SIV vaccine (139, 140) could undergo reversion to the wild type. Continuing studies in neonates and adult macaques vaccinated with live attenuated SIV vaccines confirm that this virus can cause AIDS in some neonates and adults (141). Notwithstanding these reservations, the experiments with the SIV model have presented the most tangible indication that some form of vaccine for HIV-1 may be feasible.

The evidence from human studies in support of immunity from AIDS is mostly anecdotal and controversial in some respects. Rowland-Jones et al. (142) have reported the presence of HIV-1 specific cytotoxic T cells in HIV-exposed but uninfected Gambian women. Their CTL activity may represent a protective immunity against HIV infection. Several studies have described prenatally exposed infants who had apparently cleared the virus (143-145). In addition, Clerici and colleagues (146) have shown that lymphocytes from 8 of 20 uninfected infants (40%) respond to the viral gp120 peptide by producing interleukin 2 (IL-2) in culture. These findings suggested that the presence of high titers of maternal neutralizing antibodies correlate with lower rates of vertical transmission of HIV-1 (143-146). Yet other investigators have presented a contrary view of the immunizing effects of neutralizing antibodies in prenatal infection (147, 148). Several reports have suggested that subjects with high titers of neutralizing antibodies to the homologous strain exhibit a lower rate of progression to clinical disease than those with low titers of such antibodies (149, 150). Furthermore, preliminary results from a study to evaluate the safety and immunogenicity of the MN-derived recombinant gp120 as a vaccine prototype indicated that

the vaccine induced antibodies that neutralize the homologous strain (54, 151). However, these antibodies were ineffective when tested against primary field isolates (54, 151, 152).

One important component of a protective immune response appears to be neutralizing antibodies directed against the variable linear epitopes and discontinuous antigenic sites in gp120 (153, 154). Specifically, two predominant neutralizing sites have been identified in gp120. A highly variable, subtype-specific principal neutralizing determinant (PND) is located between the two cysteine residues at positions 308-336 that constitute the V3 loop (153-158). Antibodies to this region were initially demonstrated to be type specific in their neutralizing properties and more cross-reactive when examined by peptide ELISA (59, 155, 159) although more broadly neutralizing antibodies to V3 loop have also been observed (160). Substitutions within the PND sequences, particularly at the crown of the V3 loop have been shown to exert profound effects on viral infectivity, tropism and syncytium formation (82-85, 153, 161). The DNA fragment that encodes the V3 loop and PND is the most variable sequence in the HIV-1 proviral genome (reviewed in 36). A more broadly neutralizing conformational epitope has been recently mapped to the CD4-binding region (162-166). In HIV-1 infected patients, most of the neutralizing antibodies are directed against the CD4 binding site and they block the gp120-CD4 interaction (210). Recently Wyatt et al. (68) have generated the X-ray crystal structure of a gp120 core/two domain CD4/ 17b Fab complex. This complex will provide a framework for visualizing important interactions between HIV-1 and the immune system. In addition, several previously uncharacterized antigenic sites in gp120 have been identified in our laboratory using computer-assisted epitope mapping and serological methods (121-123).

The objective of this study is to assess the impact of sequence variation on the immunogenicity and antigenic specificities of the novel field isolates of the subtype B virus. A region of ENV encoding the C2 to V5 domain has been amplified from viral genomic DNA present in the peripheral blood leukocytes (PBLs) of infected individuals. This region

DNA present in the peripheral blood leukocytes (PBLs) of infected individuals. This region was selected for this study since it encodes the PND located in the V3 loop and the chemokine receptor binding residues and critical residues involved in gp120/CD4 binding (reviewed in Reference 11). The second objective of the study is to examine the possible impact of genetic variation on the distribution of linear antigenic B-cell epitopes in gp120. A computer-assisted analysis of the predicted C2-V5 domain of the virus was compared to that of the other North American and African isolates to determine the probable influences of sequence variation on epitope distribution and immunogenicity of gp120 encoded in field isolates. The proviral DNA clones and MAPs comprising of the V3 loop sequences was used in a plasmid and peptide immunization protocol, respectively to define the immunogenity and antigenic specificity of the antibodies and CMI responses elicited by the respective rgp120 peptides. The use of these reagents in extended studies could possibly complement other efforts to identify and develop broadly protective candidate immunogens for HIV-1.

MATERIALS AND METHODS

Source of test sera and viral specimens. ARTC maintains an enrollment of more than 2,000 IVDUs patients in six ambulatory methadone maintenance clinics located in five distinct suburbs of Brooklyn and Manhattan in New York City. Participants in this study were IVDUs who tested seropositive for HIV in ELISA. Leukocytes recovered from whole peripheral blood were used as the source of proviral DNA (123).

Preparation of Proviral DNA samples. PBLs were recovered from the buffy coats using ficoll-hypaque density gradient centrifugation as described (169). Proviral DNA was purified from detergent lysates of the leukocyte pellets using a DNA/RNA isolation kit (USB, Ohio) and then subjected to PCR using nested primer pairs (Table 1).

A 683 bp fragment of ENV was amplified. This segment of the ENV encodes the region extending from the C2 to the V5 in gp120 (Figure 6). The selected region comprises the PND located in the V3 loop, a putative CD4 binding site, and five other linear antigenic epitopes (123). This segment of ENV is suitable for the generation of reliable phylogenetic trees for the determination of the various clades of HIV-1 (120-123, 168).

Table 1. ENV oligonucleotides used as primers for PCR.

Primer	**Sequence**	**Location (pNL43strain)**
P5-2 (upstream)	5'-CCAATTCCCATACATTATTTGT-3	6848-6868
P2 (downstream)	5'-GACGCTGCGCCCATAGTGCTTCC TG-3	7815-7789
P5 (upstream)	5'ACACATGGAATTCGGCCAGT AGT-3	6957-6978
P4-3 (downstream)	5'-ATTCACTTCTAGAATTGTCCCTC-3	7661-7639

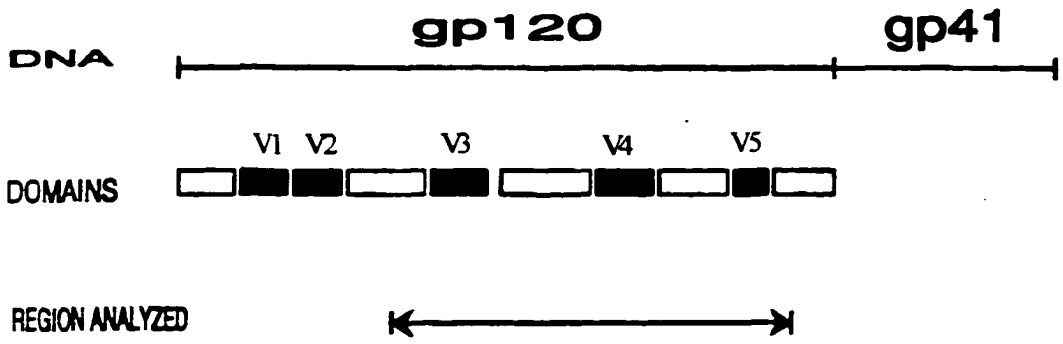


Figure 6. A linear representation of ENV showing the relative locations of the encoded V3 loop in gp120, which were amplified using PCR. The variable regions are shaded while the constant regions are not shaded.

Polymerase chain reaction (PCR). All PCR reactions were performed in the Perkin-Elmer model- thermocyler (model 9600). Nested PCR was conducted using the primers listed in Table 1. Five µl of the purified DNA (~500 ng) was added to 45 µl of a reaction mix [10 mM Tris-HCl (pH 8. 3), 50 mM KCl, 200 µM of each of the four deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP), 25 mM $MgCl_2$, 30 pmol of each primer (Keystone, CA), distilled water and 1. 5 units of Ampli- Taq (Applied Biosystems/ Perkin-Elmer, CA)]. The samples were covered with 50 µl of light mineral oil (Sigma, MO) prior to temperature cycling. The samples were first heated at 95°C for 5 min to denature the DNA templates. Amplification conditions for the first stage PCR were as follows: 95°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec. The cycles were repeated 35 times with a final extension at 72°C for 10 min. For the second stage PCR, 5 µl of the first PCR product was

added to 45 µl of the new reaction mixture and amplification was conducted under the same conditions as described above. DNA products were examined under standard electrophoresis conditions using 1. 0 % agarose gels (FMC, Bioproduct, ME).

Cloning and sequencing of PCR products. The PCR products were analyzed using 1% low melting point agarose gel. The ethidium bromide-stained bands were recovered and digested with Gelase (Epicenter, Inc. WI) at 45°C overnight. Following overnight incubation. the PCR products were precipitated with 5M ammonium acetate (Sigma) and ethanol (Sigma) and then cloned into the pT7 Blue vector (Novagen, WI) (Figure 7) at a ratio of 1:1 utilizing the 'A' overhangs produced by Taq DNA polymerase during the PCR reaction. The pT7 Blue vector carries dT overhangs that facilitate direct ligation of PCR products.

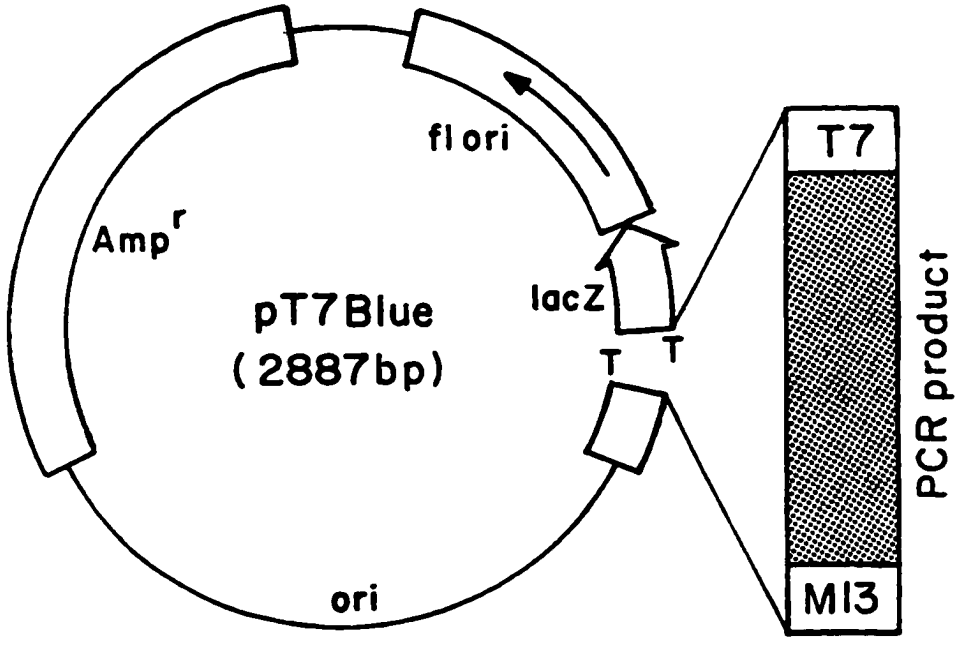


Figure 7. Map of pT7 Blue vector (Novagen) used for the ligation of the PCR products.

Transformation of E. coli. Five micoliters samples of the ligation mix were used to transform Nova Blue competent E. coli cells (Novagen) in 100 µl of SOC medium (Novagen). The β-gal negative transformants were selected on X-gal plates after overnight incubation at 37°C. The positive recombinant (white) colonies were further screened by the use of PCR using the M13 forward and T7 primers (Table 2), which bind to sequences that flank the cloning site in pT7 (Figure 6); this step was performed to determine the presence of viral DNA inserts of the correct size. DNA samples determined to be positive were purified using the alkaline lysis method (167).

DNA sequencing. The double stranded DNA plasmids were sequenced in both directions by the dideoxynucleotide chain termination method (169) using 35dATP and the Sequenase kit (version 2. 0, USB/ Amersham). Initially, the IP1 and IP2 primers (Table 2) were used to sequence the V3 loop. The inserts were sequenced from both directions using M13 forward and T7 primers (Table 2) that flank the polylinker site in the vector. The sequence was extended throughout the entire fragment using the primer walking method (119). The sequencing reactions were analyzed on 6% denaturing polyarcylamide gel (167). The wet gel was transferred to 3 mm filter paper (Whatman, OR), vacuum dried and then autoradiographed. In subsequent experiments an automated DNA sequencing protocol was instituted. The plasmid constructs were then sequenced with an automated DNA sequencer using Taq DNA polymerase and fluorescent dye-deoxynucleotide terminators (ABI Sequencer model 373A and the Prism kit). The double stranded DNA templates (1 µg per reaction) were added to a reaction mixture (Perkin Elmer) containing Taq polymerase, nucleotide dye-terminators, sequencing buffer and 30 pmol of the selected primer. The reaction mixtures were then heated at 95°C for 1 min to denature the DNA and was then subjected to cycle sequencing using the following temperature cycle: denaturation at 95°C for sec and annealing/extension at 50°C for 4 min. The cycles were repeated 25 times. CentriSep 100 spin columns (Princeton Separations, NJ) were used to purify the resulting

products from the unincorporated primers and dye-terminators. The reactions were analyzed on 6 % denaturing polyacrylamide gels. Base calling and data processing were conducted with Seqed (version 1. 0, Applied Biosystems). All nucleotide sequences have been submitted to GenBank under the following accession numbers: U11586 through U51199.

Table 2. Oligonucleotides used as sequencing primers. Nucleotides in parentheses are degenerate primer positions.

Primer	Sequence	Location (strain)
Upstream (sense)		
BD1	5'TTGT(A/G)(A/G)GG(A/G)GAATTTTTCA	6900-6921 (IIIB)
IP1	5'AAGAAGAGGTAGTAATTAGAT	6570-6590 (IIIB)
OP1	5'ATGTCAG(C/T)ACAGTACAATGTACAC	6491-6515 (IIIB)
M13 forward	5'GTAAAACGACGGCCAGT	
Downstream (antisense)		
IP2	5'AT(A/C)TGGGTCCCCTCC(A/T)GAGGA	6860-6879 (IIIB)
OP2	5'TAGAAAAATTCCCCTCCACAA	6900-6921 (IIIB)

Phylogenetic Tree Analysis. Phylogenetic analysis was conducted over the 625 bp region of the ENV gene (GP120). The alignment of the nucleotide sequences was conducted using the CLUSTAL program in PC\Gene program package (version 6. 7, Intelligenetics, CA). Phylogenetic trees were constructed using the neighbor-joining method, and confirmed with the parsimony and the UPGMA tree algorithms in MEGA (version 1. 01) (170). The trees were rooted by the CIV_{gab} outgroup since the true ancestor of HIV-1 is still unknown. Confidence limits for the nucleotide clusters were generated using bootstrap analysis (137). The BOOTSTRAP test generated these confidence limits by sampling each set of the sequence data 500 times. Gaps introduced to permit sequence alignment were removed prior to phylogenetic analysis. The sequences of the reference isolates, MN, NOF,

and U455 were retrieved from GenBank. The reference sequences were selected to allow ENV clustering according to the Los Alamos National Laboratory nomenclature (97).

Prediction of linear antigenic epitopes in gp120. Amino acid translations were performed using PC\Gene and positions were numbered according to the sequence in $IIIB_{HXB2R}$ strain (96). Linear antigenic epitopes were determined using the algorithms in SURFACE PLOT (version 1.4) and POLAR TRIPEPTIDE in PPSP (Synthetic Peptides Inc., Alberta, Canada), and PEPTIDE STRUCTURE in GCG (version 8) (171-173). SURFACE PLOT predicts surface and interior sites for tripeptide residues in proteins using a composite value for hydrophilicity, flexibility, and accessibility (171). POLAR TRIPEPTIDE determines high probability surface residues utilizing values for surface exposure obtained from the Brookhaven Database of protein X-ray crystallographic structures (171). High (60%) and intermediate (25%) probability cutoffs were used to generate antigenic residues reported herein. PEPTIDE STRUCTURE utilizes the Jameson-Wolf algorithm to derive a combined value for antigenicity based on hydrophilicity, flexibility, surface probability, and secondary structure parameters (β turns, coils, β sheets and α helices) (173). Regions predicted to contain three or more contiguous "hidden" residues are not considered to be antigenic in these analyses.

Synthetic peptides. Peptides predicted to comprise the V3 loop epitope were synthesized using the solid-phase procedure (174) on an automated peptide synthesizer (Synergy, Model 432A, Applied Biosystems, CA) with fmoc (9-fluromethoxycarbonyl) protected amino acids, and hydroxymethylphenyloacetic resin as described (174). Post-synthesis cleavage from the resin and protecting groups was conducted using an aqueous solution containing the scavengers: thioanisole, trifluoroacetic acid, and dithioethane. The peptides were filtered through glass-wool, precipitated in cold methyltert-butyl ether (MTBE, Sigma), and extracted with aqueous acetic acid. Three more washes of the solubilized peptide were performed using methyltert-butyl ether to remove any traces of contaminants.

Further purification of the peptides has not been found necessary for reactivity in enzyme linked immunoasborant assay (ELISA) (96, 122). The sequences for the monomeric peptides are given (Table 3). MAPs (Figure 8) were synthesized on a Fmoc-MAP-4 Branch Resin and then cleaved and purified in the same manner as the linear peptides. MAPs initially described by Tam et al. (175, 176) have demonstrated several advantages in producing anti-peptides antibodies. MAPs typically have 4 or 8 peptide arms branching from a lysine core matrix. Because of the molecular complexity of structural design there is no need to conjugate the peptide to a carrier protein immunogen.

Table 3. V3 loop peptides tested in ELISA.

Subtype of HIV-1	**V3 Amino acid sequence**	**length**
B_RT1 con	RPNNNTRKGIHIGPWGTFFA	19
B_RT3.6	RPSMNTSKGIHIGRAFYTTEAITGDIR	29
B_RT3.15	RPSMNT**R**KGIHIGRAFYTT**GEII**GDIR	29
B_RT1.4	RPNNNTRKGIHIGPWGTFFATGNIIGD	29
A_UG06c	RPYKVRRRKHIGPGRSFYT	19
C_UG045	RPNNNTRESVRIGPGRAFY	19
D_UG23c	RPYENVRHRTPIGLGQALI	19

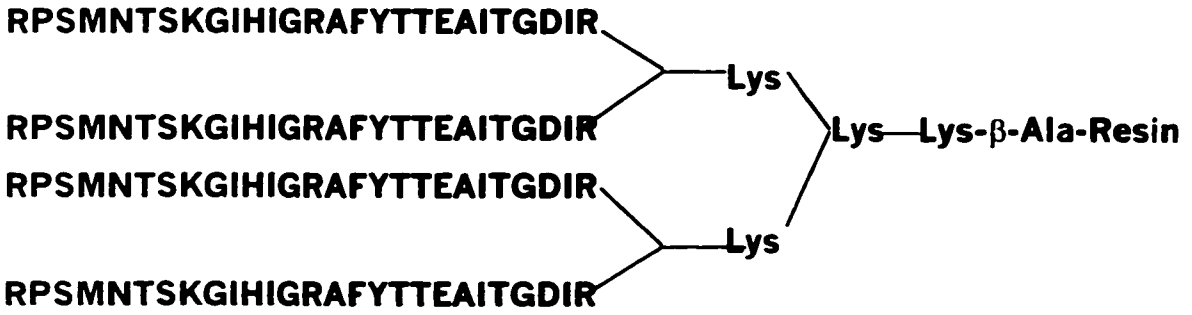


Figure 8. Schematic representative of one of the MAPs used in this study. The sequence shown in this example corresponds to that of the V3 loop derived from clone B_RT3.6.

ELISA. ELISA was conducted as previously described (177). Ninety six-well polyvinyl chloride microtiter plates (Baxter Scientific, Edison, NJ) were sensitized with 125 µl of peptides at a concentration of 50 µg/ml of 0. 1 M $NaHCO_3$ (pH 9. 6) for 24 hours at 4°C. This concentration of the antigen was shown to yield the best discrimination (>8-fold difference) between test and control assay samples in preliminary studies.

Duplicate or triplicate assays were performed for each peptide. After seven washes with ELISA buffer [15 mM Tris, 75 mM NaCl, pH 7. 2 and 0. 1 % Tween 20], the wells were incubated with 125 µl of blocking buffer [ELISA buffer containing 1/50 dilution of normal goat serum (NGS) and 0. 4 % BSA] for 1 hr at 37°C. The buffer was discarded and replaced directly with 125µl of the test serum diluted 1/50 in fresh blocking buffer. Sera were incubated with the antigen for 1 hr at 37°C and the plates were washed seven times with ELISA buffer. Peroxidase-labeled polyvalent goat anti-human whole IgG (Cappell, CA) was diluted 1/2500 in conjugate buffer (ELISA buffer containing 1/250 NGS and 0. 08

% BSA) (177). The plates were incubated with 125 µl of the conjugate for 1 hr at 37°C and then washed seven more times. The peroxidase substrate was prepared by dissolving 6 mg of o-Phenylenediamine Dihydrochloride (OPD) (Sigma) and 1 tablet of urea H_2O_2 (Sigma, MO) in 15 ml of substrate buffer [10 mM Tris, 50 mM NaCl, and 1 mM EDTA, (pH 3. 2)]. The substrate (125µl) was added to each well and reacted for no longer than 30 minutes. The reactions were stopped by the addition of 50µl of 4 M H_2SO_4. All O. D. readings were recorded at 490 nm in a microplate spectrophotometer (model 550, BioRad, CA). Normal sera from New York donors were included in all assays as negative controls. The O. D. readings from these tests were $\leq$ 5% of the experimental values. The background readings were subtracted from the experimental values to facilitate data comparison.

Construction of expression plasmids for DNA immunization. The plasmid, CMVint-BL. was previously constructed by Chapman et al. (178) and subsequently modified to include several additional cloning sites (Figure 9). The vector contains the SV40 origin of replication. human cytomegalovirus (Towne strain) major immediate-early gene enhancer/promoter (hCMV IE1) and intron A (178-182). The hCMV IE1 promoter is among the most efficient viral gene regulators available for transient expression of diverse heterologous proteins (182). The versatility in the performance of hCMV IE1 has been attributed to the function of a number of motifs in the DNA sequences upstream of the promoter which include multiple potential binding sites for many well known eurkaryotic transcriptional control factors such as nuclear factor 1 (NF1). These factors are present in cells of diverse tissue origin including muscle (182). The human tissue plasminogen activator (TPA) signal sequence present in CMVint-BL has been shown to enhance the secretion of several reporter heterologous glycoproteins including gp120 (183, 184).

The ENV gene (625 bp) was PCR-amplified from the existing PT7 plasmid using the primers listed (Table 3). The 5' primers were designed to exclude the gp120 transcription start codon and signal sequence (178). This cloning strategy was based on experimental

evidence that the functions of the gp120 start codon and or signal peptide is inhibited in the absence of rev (178). The PCR products were then ligated into the BamH1 site in the CMV-int/BL vector using standard restriction and cloning procedures (167). The in-frame ligation and the orientation of the inserts was confirmed by restriction enzyme analysis and DNA sequencing using the automated DNA sequencing protocol (above). The CMV-int/BL constructs of the ENV gene from clones B_RT3.6, B_RT3.15 and B_RT1.4 were designated: CMV/B_RT3.6. CMV/B_RT3.15. and CMV/B_RT1.4, respectively.

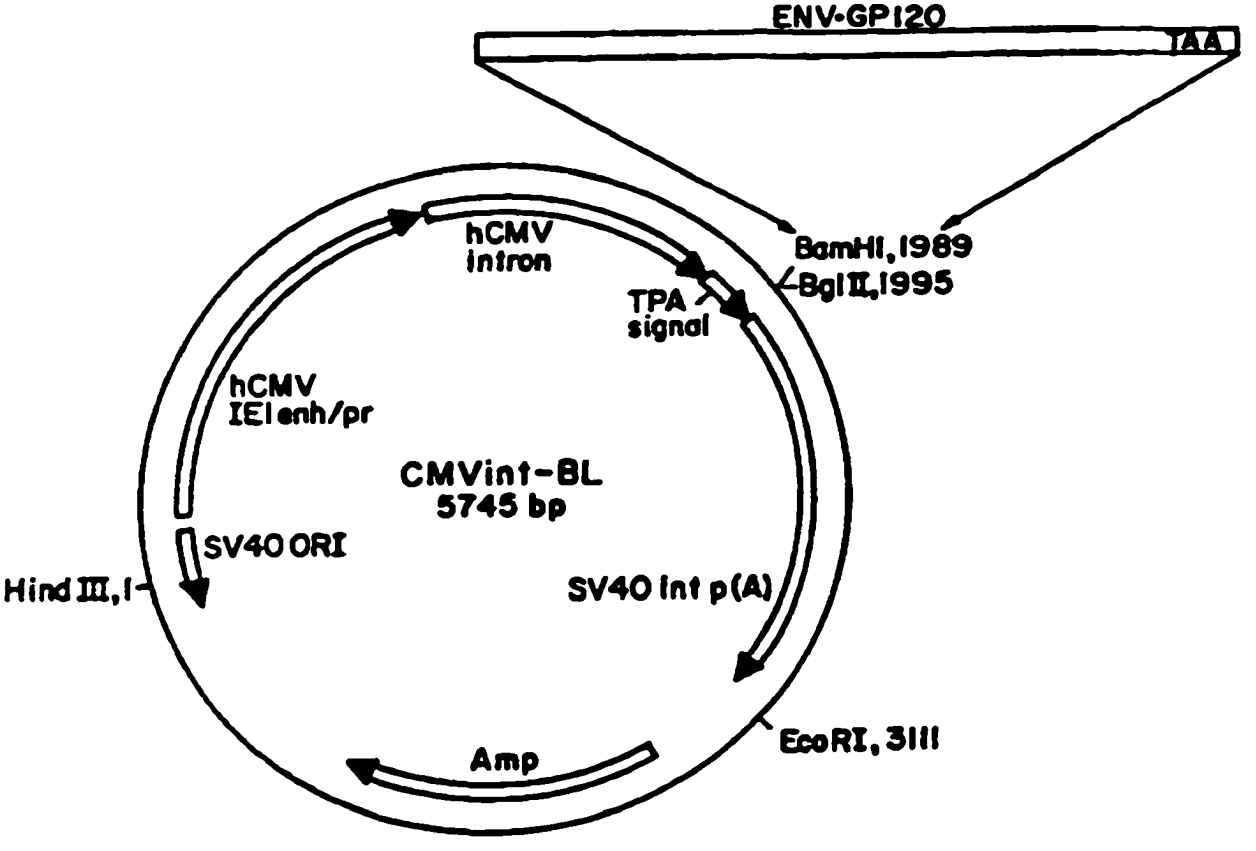


Figure 9. Map of CMV int-BL vector used for ENV expression and DNA-mediated immunization. (Adapted from reference 184).

Table 4. Oligonucleotides used as PCR primers for ENV. The PCR primers were designed to include the Bam HI site (underlined) and stop codon (bold).

Primer	**Sequence**	Location (strain)
Upstream (sense)		
B_RT3.6c	5`CCTGGATCCCTCACAAACAATG**CTA**	6857-6878 (IIIB)
B_RT1.4	5`TTGGGATCCAGTCTAGCAGAAGAA**GAG**	
Downstream (antisense)		
B_RT3.6	5`CTGATTCCTCGATACAAGACTGGATCC**AAC**	7495-7517 (IIIB)
B_RT1.4	5`CTCTATATCTCCCTGTTAATCGGATCC**CTT**	

In vitro expression of rgp120. To investigate the efficiency of the constructs to express gp120, COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4. 5 mg/ml glucose and 10% fetal bovine serum at 37°C in 5% CO_2/air. Cells were seeded into 6 well culture plates with $2X10^5$ cells per well prior to transfection, and cultured overnight. Transfection was performed the following day by using Lipofectin reagent (GIBCO. BRL) as instructed by the manufacturer. The DNA transfection mixture comprised 2 μg of the various ENV constructs, or the control expression vector. The cultures was incubated for a maximum of 72 hours at 37°C in 5 % CO_2 and 95 % humidity. Twenty four, 48 and 72 hours after transfection, cells were analyzed by ELISA and RT PCR.

Cells were harvested at 24 hour intervals, lysed, and the protein extracts were then quantified by O. D. measurement at 600 nm using the modifications of the Bradford method as described in the Quantify Protein Assay System (Pierce, USA). Expression of the

recombinant proteins was detected using ELISA as described above. One hundred twenty five µl of the cell extract at a protein concentration of 50 µg/ml in 0. 1 M $NaHCO_3$ (pH 9. 6) was used to sensitize 96-well polyvinyl microtiter plates (Baxter Scientific, NJ) overnight at 4°C. Antibody reactivity of the cell extracts was determined using previously characterized hyperimmune serum samples from HIV-1 infected Ugandan donors. Peroxidase labeled goat anti-human whole IgG (Sigma) was used as the indicator reagent. Background values were obtained by reacting normal human sera simultaneously with the test samples. The negative control values were subtracted from the experimental values to facilitate sample to sample comparison.

Quantitation of ENV expression by Reverse Transcription Polymerase chain reaction (RT-PCR). All PCR reactions were performed in the Perkin-Elmer model- thermocyler (model 9600). DNA-transfected cells were harvested at 24 hr intervals, and RNA was isolated using RNAzol (Gibco) according to the manufacturer's protocol. The pellet was then dissolved in 30 µl of DEPC treated water. RT-PCR was then conducted as follows: The first-strand cDNA synthesis was performed in a 20 µl reaction containing 4 µl 5X RT buffer (Gibco), 2 µl 0.1M DTT, 2 µl 10mM NTP mix (Pharmacia), 500ng oligo(dT), (Gibco), 1 µl RNasin (Gibco), 1 µl Reverse Transcriptase (Gibco) and 10 µl RNA (~5µg). Samples were incubated at 42°C for 1 hour. The reaction was then heated at 70°C for 10 min to inactivate the enzyme. Nested PCR was conducted using the primers listed in Table 4 and for β-actin the following PCR primer pair: upstream 5'- CGTGGGCCGCCCTAGGCACCA, downstream 5'-TTGGCCTTAGGGTTCA GGGGGG). Five µl of the cDNA was added to 45 µl of a reaction mix [10 mM Tris-HCl (pH 8. 3), 50 mM KCl, 200 mM of each of the four deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP), 25 mM $MgCl_2$, 30 pmol of each

primer (Keystone, CA), distilled H_2O and 1. 5 unit of Ampli- Taq (Perkin-Elmer Cetus, CA)]. The samples were covered with 50µl of mineral oil (Sigma, MO) prior to temperature cycling. The samples were first heated at 95°C for 5 min to denature the DNA. Amplification conditions for the regular PCR were as follows: 95°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec for 35 cycles with a final extension at 72°C for 10 min. DNA products were examined under standard electrophoresis conditions using 1.5 % agarose gels (FMC, Bioproduct, ME).

DNA Immunizations. Female BALB/c mice, 4 to 6 weeks old, were obtained from Jackson Laboratory (Bar Harbor, ME). Groups of 3-6 mice were inoculated into the quadriceps muscle with 100 µg of the plasmid in 200 µl of saline (184). The animals were boosted at defined time intervals (2 weeks) and control mice were inoculated with CMV-INT/BL without the ENV insert. Serum samples were obtained from 100-300 µl of blood collected from the eye vein at one month intervals. The blood samples were allowed to clot overnight at 4°C and then centrifuged at 10,000 rpm for 10 min. The serum samples were stored at –20°C until assayed.

MAPs Immunizations. Female BALB/c mice, 4 to 6 weeks old, were obtained from Jackson Laboratory (Bar Harbor, ME). Groups of 3-6 mice were inoculated in the quadriceps muscle with 15-50 µg of the MAPs and 1 µg of GERBU adjuvant (Valley Center, CA) in 200 µl of saline (184). The animals were boosted at defined time intervals (2 weeks) and control mice were inoculated with adjuvant alone. Serum samples were obtained from 100-300 µl of blood collected from the eye vein using a capillary at 2 weeks intervals and then processed as above.

In vivo expression of ENV plasmids by RT-PCR. RT-PCR was performed for the ENV gene as described in the mRNA capture and RT-PCR kits (Boehringer Mannheim, IN). To assess the in vivo expression of ENV, the DNA-inoculated quadriceps muscle of the mice was dissected and ground into powder by using pre-cooled mortar and pestle on dry ice. The powder (~0.4 g) was resuspended in 4 ml of lysis buffer and then homogenized. The homogenate (800 µl) was spun for 30 sec, and 20 µl of oligo $(dt)_{20}$ working solution was added, and the sample was incubated for 6-9 min at 4°C. Then 50 µl of the mix were added to the streptavidin-coated tube and incubated for 6 min at 4°C. The mix was removed and the tube was washed three times. The captured mRNA preparation was then subjected to RT-PCR. RT-PCR was performed using a kit (Titan One Tube RT-PCR System, Boehringer Mannheim) and the following primer set was used (Table 4) and for β-actin the following PCR primer pair was used: upstream 5'-CTACAATGAGCTGCGTGTGG, downstream 5'-AAGGAAGGCTGGAAGAGTGC(R&D Systems, MN). 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 product was analyzed in 1.5 % agarose gel.

ELISA for mouse anti-gp120 antibodies. ELISA was conducted essentially as described above for human antibodies with some modifications. The ELISA plates were blocked with 125 µl of 1 % BSA (Boehringer Mannheim) in PBS [137 mM NaCl, 2. 7 mM KCl, 10 mM Na_2HPO_4 and 1. 8 mM KH_2PO_4] (pH 7. 4) for 1 hr at 4°C. After one hour the plates then were washed with 150 µl of PBSTB [PBS containing 0. 05 % Tween 20

(Sigma) and 1 % BSA] and 125 µl of the test serum diluted 1/50 in PBSTB was then added to the wells. The plates were incubated for 1 hr at 4° C. The wells were washed 10 times with 200 µl of PBSTB and then incubated for 1 hr at 4 °C with 125 µl (33 ng/ml) of horseradish peroxidase-labeled goat anti-mouse whole IgG (Promega, WI) diluted in PBSTB. Sera from the control mice inoculated with CMVint-BL without the ENV insert was analyzed simultaneously to provide background values.

Proliferation assay. Splenocytes were aseptically removed from the mice and teased in sterile RPMI 1640 medium. The cells were then washed twice with the culture medium supplemented with 2 mM glutamine and 20 mM HEPES. The number of splenocytes was adjusted to $2x10^6$ cells/ml in complete T cell medium [equal volumes of RPMI 1640 and EHAA (Gibco BRL, NY), 10% fetal bovine serum, $5x10^{-5}$ M mercaptoethanol (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin]. Two hundred µl of the cell suspension was cultured in triplicate in 96-well microculture plates with the respective peptides (50 µg/ml) or PHA (5 µg/ml, Sigma), for three days at 37 °C in 5 % CO_2 and 95 % humidity. The cells were then pulsed by the addition of 1 µCi of ^{3}H-Thymidine (^{3}H- TdR) (DuPont, DE) for 6 hr. Radiolabeled DNA was harvested on glass fiber filter paper after the pulsed cells were lysed with 1% Triton X 100. The samples were then air-dried and the isotope was quantified using a liquid scintillation counter. ^{3}H-Thymidine incorporation was then expressed in counts per minute (CPM).

Cytokine production by stimulated splenocytes. Splenocytes were aseptically removed from the mice and teased in sterile culture medium. The cells were then washed twice and prepared for culture as above. The culture supernatants were removed and stored

at -70°C for cytokine measurement. Spontaneous released of IL-2 was determined by using commercially available kit (Endogen, Worcester, MA). ELISA was performed in duplicate for each test sample. O. D. readings were determined at 450 nm and the mean values were calculated.

RESULTS

Isolation of ENV sibling clones from isolates RT3 and RT1. This study summarizes the results of the experiments performed on the sibling clones of the ENV gene previously amplified from the two patient isolates: RT1 and RT3. Proviral DNA samples were amplified from the leukocyte pellets of the donors. Nested PCR yielded 683 bp fragments (Figure 10). The subcloned fragment of the ENV gene encodes the sequence extending from the C2-V5 domain of gp120. This region of gp120 encodes several structural and functional domains, which include the immunogenic V3 loop, CTL epitopes, CD4 and chemokine receptor binding sites, and a number of previously uncharacterized antigenic epitopes (123).

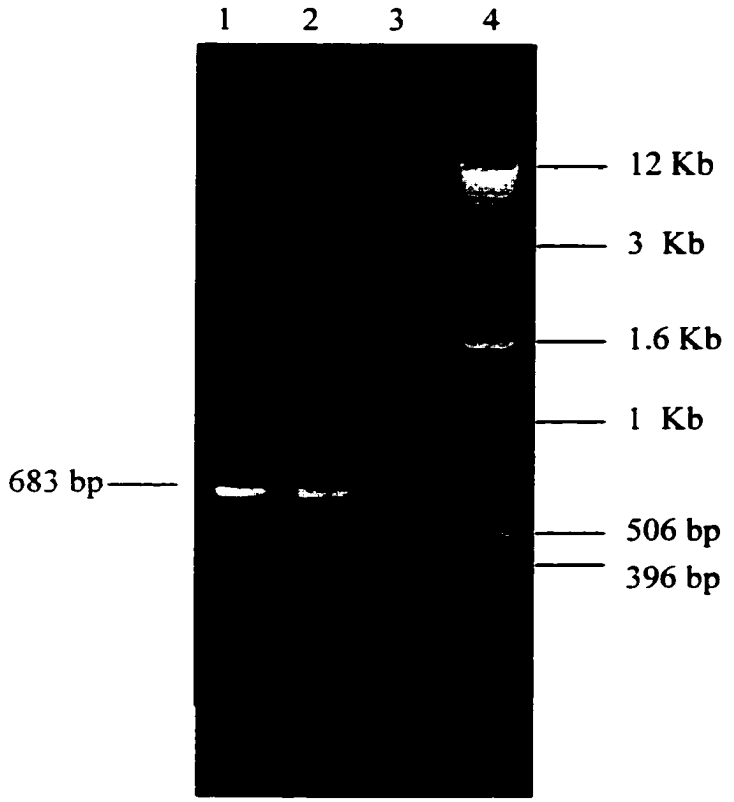


Figure 10. Ethidium bromide-stained agarose gel showing PCR amplified products from the New York isolates RT1 and RT3: RT1 (lane 1), RT3 (lane 2), negative control without DNA sample (lane 3) and DNA ladder (lane 4).

Phylogenetic tree analysis. The alignment of the nucleotide sequences was conducted using the CLUSTAL program in PC\GENE (appendix 1) and the algorithms in MEGA was used to generate a phylogenetic tree based on the ENV nucleotide sequences of the RT1 and RT3 derived clones in relation to the reference isolates MN and D_UG23c retrieved from GenBank (97, 168). The RT1 derived sibling clones B_RT1.4, B_RT1. 15, B_RT1. 17 and B_RT1. 21 clearly cluster with the members of "group B" viruses at 99% confidence level (Figure 11). Similarly, the RT3 derived sibling clones B_RT3.6, B_RT3. 11, B_RT3.12 and

B_RT3.15 all cluster with MN in group B. Thus all clones derived from these two patient isolates are assigned the letter "B" to designate their phylogenetic subtype. The assignment of RT1 and RT 3 clones to group B was confirmed by phylogenetic tree analyses of the same sequences using the maximum parsimony method (170). The "group B" virus subtypes are predominant in North America, the Caribbean and Europe (97). These analyses showed intra-isolate sequence variation of 18 % among the sibling clones B_RT3.6, B_RT3. 10, B_RT3. 11, B_RT3.12 and B_RT3.15 derived from an AIDS patient compared to a 7 % variation among the sibling clones B_RT1.4, B_RT1. 15, B_RT1. 17, and B_RT1. 21 from RT1 obtained from an asymptomatic donor. These results are consistent with other findings where low frequencies of variation were observed in the ENV sequences obtained from asymptomatic donors (98).

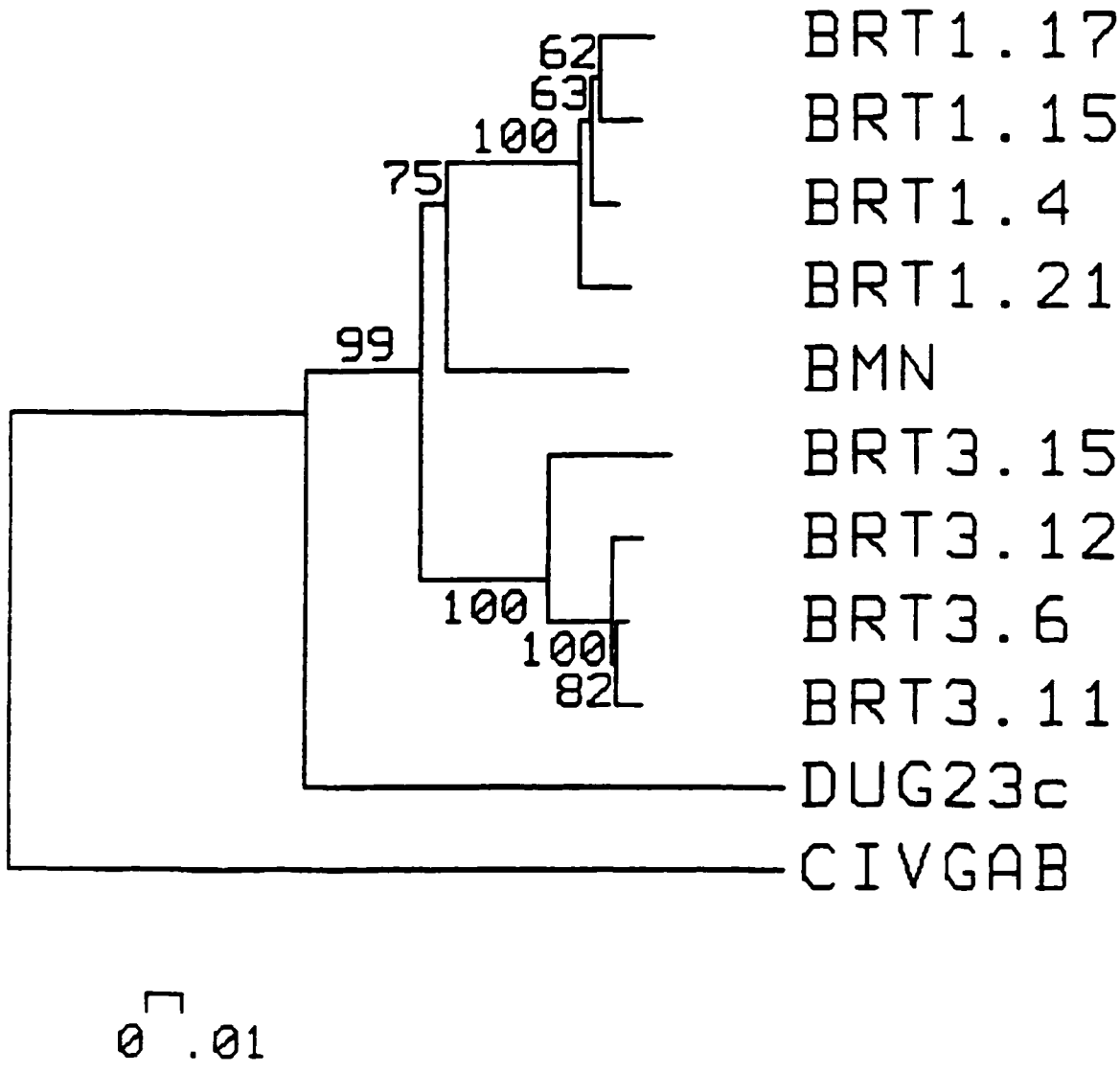


Figure 11. Phylogenetic relationships of the ENV sequences of the RT3 and RT1 sibling clones and representative HIV-1 isolates. The tree was constructed using the neighbor-joining approach of Saitou and Nei (170). The trees were rooted by the CIV_{gab} outgroup. The first letter in each sequence name indicates its cluster in accordance with Los Alamos National Laboratory nomenclature (97). The branch length indicate the degree of variation.

B_RT1 and B_RT3 clones exhibit sequence diversity in gp120. The nucleotide sequences were translated using the TRANL program in PC/GENE. The alignment of the amino acid sequences obtained from each clone is shown (Figure 12). Considerable diversity

was noted throughout the V3, V4 and V5 regions analyzed. The clones derived from isolates RT1 and RT3 displayed divergent hexapeptides at the cap of the V3 loop even though both isolates clustered in clade B. The clones derived from RT1 displayed GPWGTF, while RT3 displayed the highly conserved North American hexapeptide GPGRAF. Within the V3 loop of the RT3 derived clones the core sequence (GPGRAF) was well conserved but sequence variations were observed downstream of the cap. Specifically, the clone B_RT3.15 differed at 5 positions from the rest of the siblings (Figure 11). There were fewer amino acid sequence differences between the other variable regions of the clones derived from RT3. In comparison, the clones derived from the RT1 isolate showed little or no variation throughout the region of gp120 analyzed. No variation was found among the amino acid residues identified in HIV-1 isolates for CD4 binding site (11, 15, 16, 162, 166).

Figure 12. Distribution of linear antigenic epitopes in the B_RT3 and B_RT1 sibling clones and representative isolates. Sequences of high antigenic potential were determined according to SURFACE PLOT and PEPTIDE STRUCTURE. E1 to E8 designate the proposed epitopes. Symbols: (-) gap introduced to facilitate alignment, bold type indicates antigenic site, underlined residues were synthesized for use in ELISA.

The clones from B_RT1 and B_RT3 display analogous and divergent antigenic epitopes in gp120. Epitope mapping was conducted using a computer algorithm to access the potential impact of sequence variation on the distribution of antigenic epitopes. The SURFACE PLOT and PEPTIDE STRUCTURE analyses (121,123) displayed a high degree of correlation in their prediction of the linear antigenic residues in gp120 (Figure 12). Representative values for these predictions are shown for B_RT3.6 (Appendices 3 and 4). At least eight analogous antigenic sites, arbitrarily designated E1 to E8, were identified in B_RT1 and B_RT3 (Figure 12). Most of these epitopes were mapped to the C2, V3, V3-V4, C3 and V5 domains of gp120. The epitopes, E1, E2, E5 and E8 showed relatively conserved sequences in the regions analyzed. Analysis of the amino acid residues comprising the epitopes E3[V3-V4], E4[V3-V4], E6[V4], E7[C3] showed noticeable level of inter-isolate and intra-isolate sequence diversity and the loss of some antigenic sites. For instance, sequence variation in the region overlapping the E3 [V3-V4] site presumably caused a loss of this epitope in the RT1 clones, B_RT1.4, B_RT1.15, B_RT1.17 and B_RT1.21 compared to the RT3 clones and the reference isolate, D_UG23c (Figure 12). The variation in the primary sequences of these clones presumably resulted in the loss of E6[V4] in clone RT3.15 (Figure 12). The positions of the epitopes E2[V3], E7[C3] and E8[V5] sites are strongly consistent with the PND and the linear CD4 binding site (11, 117, 154, 155). The sequence variation observed in the PND and other regions of gp120 suggests that BRT1 and BRT3 may also differ in their reactivity with antibodies. This possibility was tested in ELISA.

The divergence in the B_RT3 clones affected the magnitudes of antigenicity as quantified using POLAR TRIPEPTIDE (Figure 13). The E6 site showed a high value of antigenicity in clone RT3.6 but lower antigenic probability in clone B_RT3.15. Despite the basic similarities in their pattern of distribution, the discontinuity in the E8 site of clone B_RT3.6 apparently reduced the predicted antigenicity compared to the E8 site in B_RT3.15.

Strong antigenic indices were predicted for the E2 [V3] site in all the isolates, with the exception of D-UG23c.

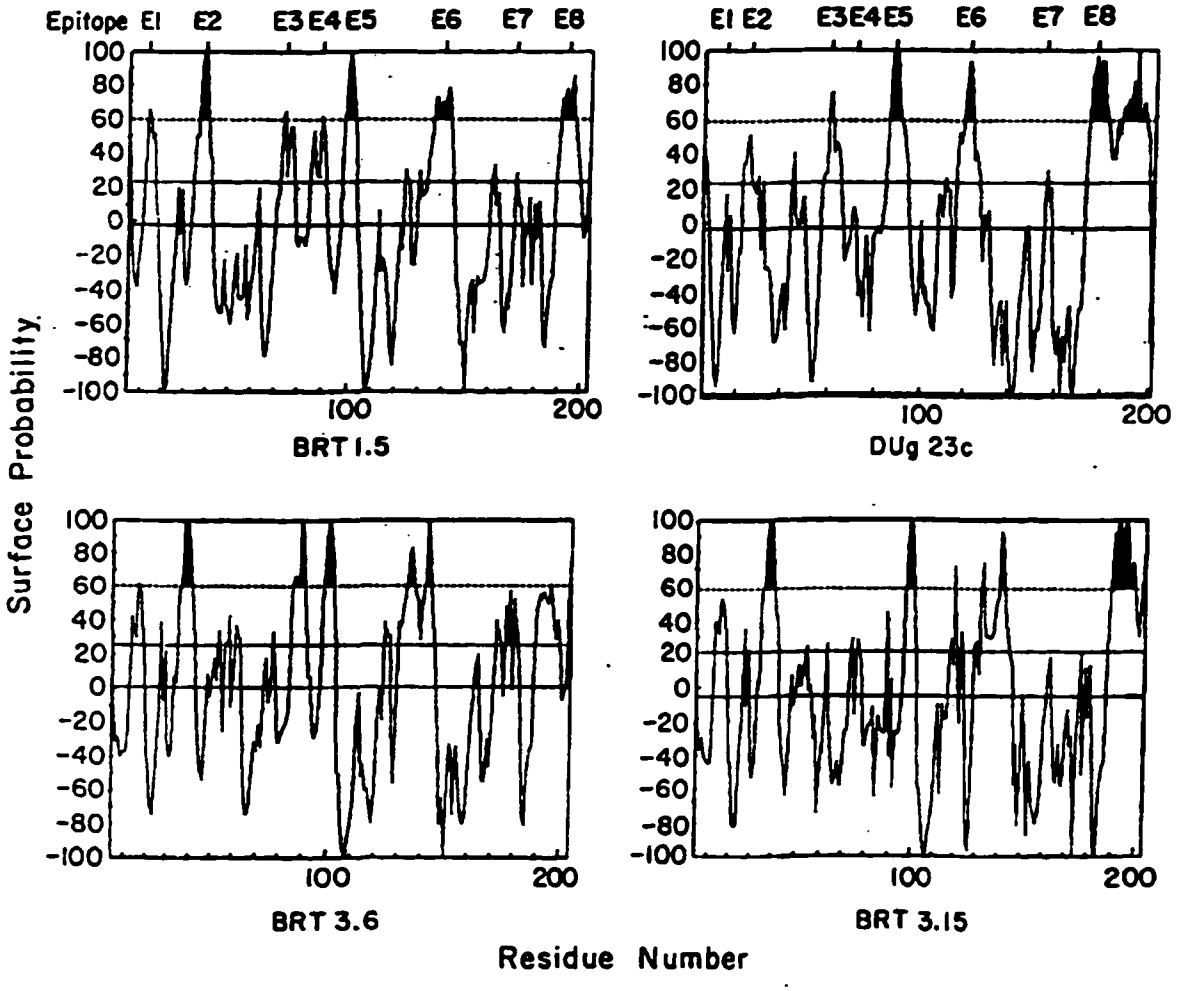


Figure 13. Surface probability profiles of gp120 encoded in the B_RT1 and B_RT3 sibling clones and a reference isolate, D-UG23c. Surface profiles were generated using POLAR TRIPEPTIDE. Residues are numbered relative to the HXB2R sequence. Shaded regions indicate locations of the major epitopes. Symbols: (. . .) indicates the intermediate cutoff values. (---) indicates the high probability cutoff values.

Variation in the seroreactivity of gp120 epitopes from BRT1 and BRT3 clones. To asses the effect of sequence variation on antibody reactivity of gp120, a total of eleven serum

samples from the IDUs cohort were tested in ELISA with synthetic peptides comprising the immunodominant E2 [V3] epitope in clones B-RT3.6, B_RT3.15, D_UG23c and the consensus BRT1 peptide (BRT1con). Reactivity of the test peptides with different sera was observed to vary in intensity (Figure 14). The reactivity of the V3 loop peptide from the RT1con with different sera was not remarkable when compared with that of the RT3 clones (Figure 14). Peptides from B_RT3.6 and B_RT3.15 reacted most intensely. These results were surprising since both the RT1 and the RT3 isolates are both subtype B viruses. The lower intensity of ELISA reactivity of the D_UG23c peptide was consistent with previously reported findings (121, 122). Even though the gp120 peptides derived from the RT3 sibling clones reacted intensely with the test sera, marked variation was noted between the molecules. For instance the ELISA reactivity of the peptide from B_RT3.6 was about two times that of the B_RT3.15 sibling. The diversity in the antibody reactivity observed against these peptides may be attributed to several factors: primary sequence variation, conformational restriction in the test antigens or the relative immunogenicity of the predicted epitopes in-vivo (185, 186). In light of the divergence in serum reactivities, a DNA-mediated immunization protocol was adapted in an attempt to elucidate a possible impact of sequence variation on the immunogenic properties of gp120 encoded by these HIV-1 field isolates.

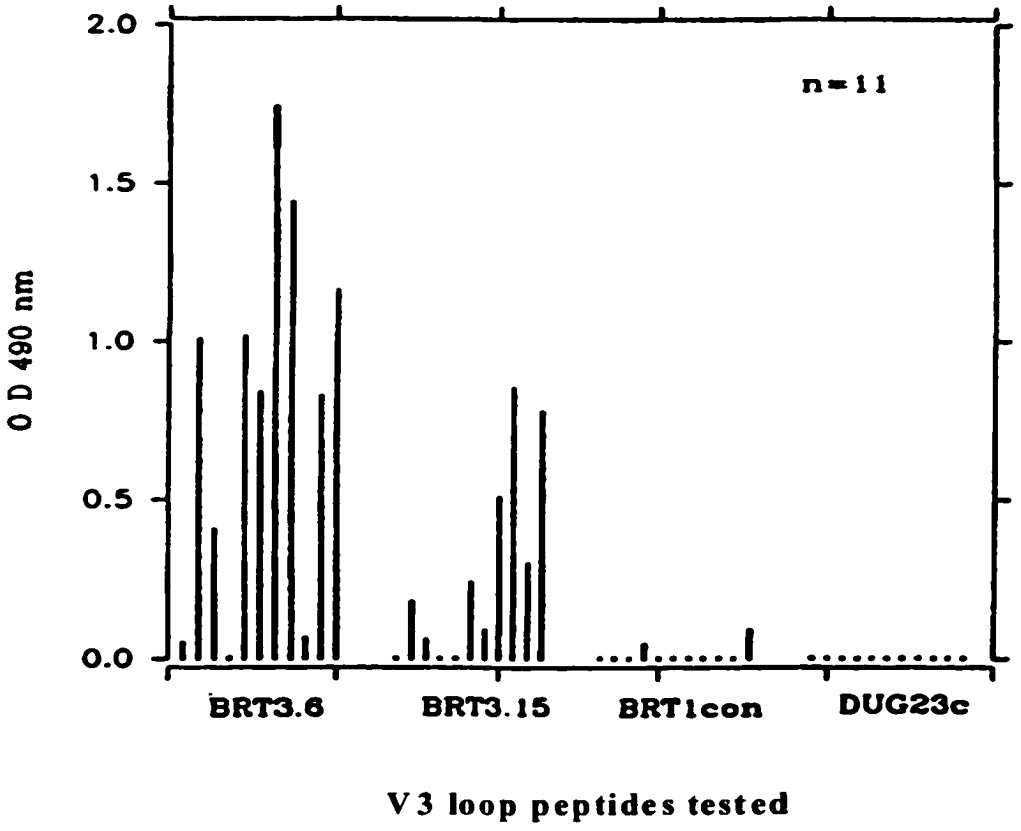


Figure 14. ELISA reactivities of serum samples from asymptomatic New York donors with synthetic peptides comprising the predicted E2[V3] epitopes of B-RT3.6, B_RT3.15, B_RT1con, and D_UG23c.

Construction of ENV expression plasmids. Direct injection of DNA into animals is a novel and promising method for delivering specific antigens for immunization (187-204). In order to determine whether DNA-mediated immunization could induce anti-HIV immune responses against the sibling clones of RT1 and RT3, the C2-V5 region of the env gene was PCR-amplified and then cloned into the CMVint-BL expression vector. The correct size

and orientation of the DNA fragment was confirmed by digestion with restriction enzymes (Figure 15) and automated DNA sequencing.

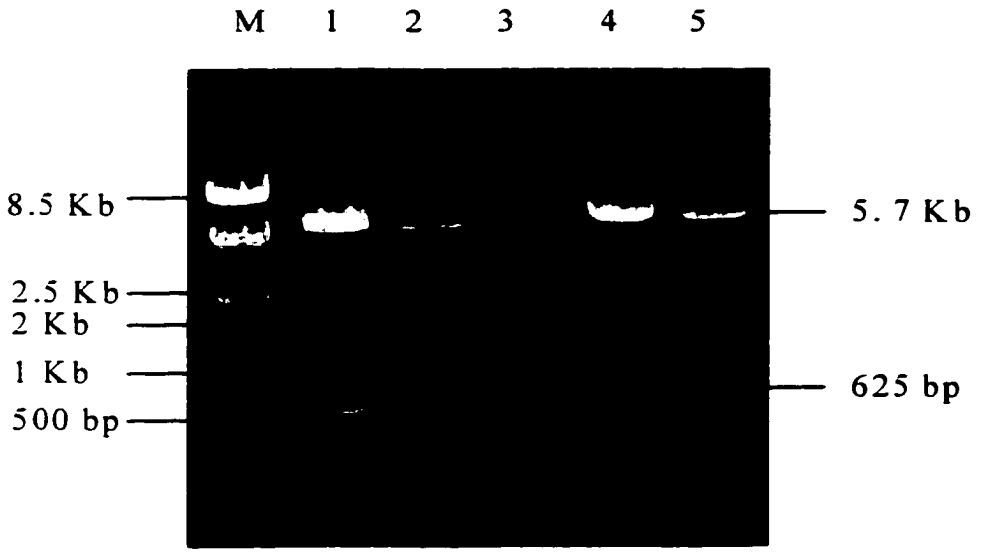


Figure 15. Agarose gel of the ENV CMV/int-BL plasmids digested with Bam HI: DNA ladder (Lane M), CMV/B-RT3.6 (lane 1), CMV/B_RT3.12 (Lane 2), CMV/int-BL only (lane 3) CMV/B-RT3.15 (lane 4) and CMV/B-RT1.4 (lane 5).

Detection of the ENV mRNA in COS-1 cells by RT-PCR. To analyze the efficiency of the plasmid constructs, COS-1 cells were transfected with 2 μg of the respective DNA. The plasmid constructs CMV/B_RT3.6, CMV/B_RT3.12, and CMV/B_RT1. 15 and CMV/B_RT1.4 were transfected into COS-1 cells. Control cells were transfected with CMVint-BL without ENV. At 72 hr post-transfection, mRNA was extracted from the cell lysate and the presence or absence of the ENV transcripts were analyzed using RT-PCR (Figure 16). Transcription was detected in the transfected cells. Control experiments using

RNA samples for the PCR assay directly detected no products in the samples indicating that DNA contamination of the samples had not occurred.

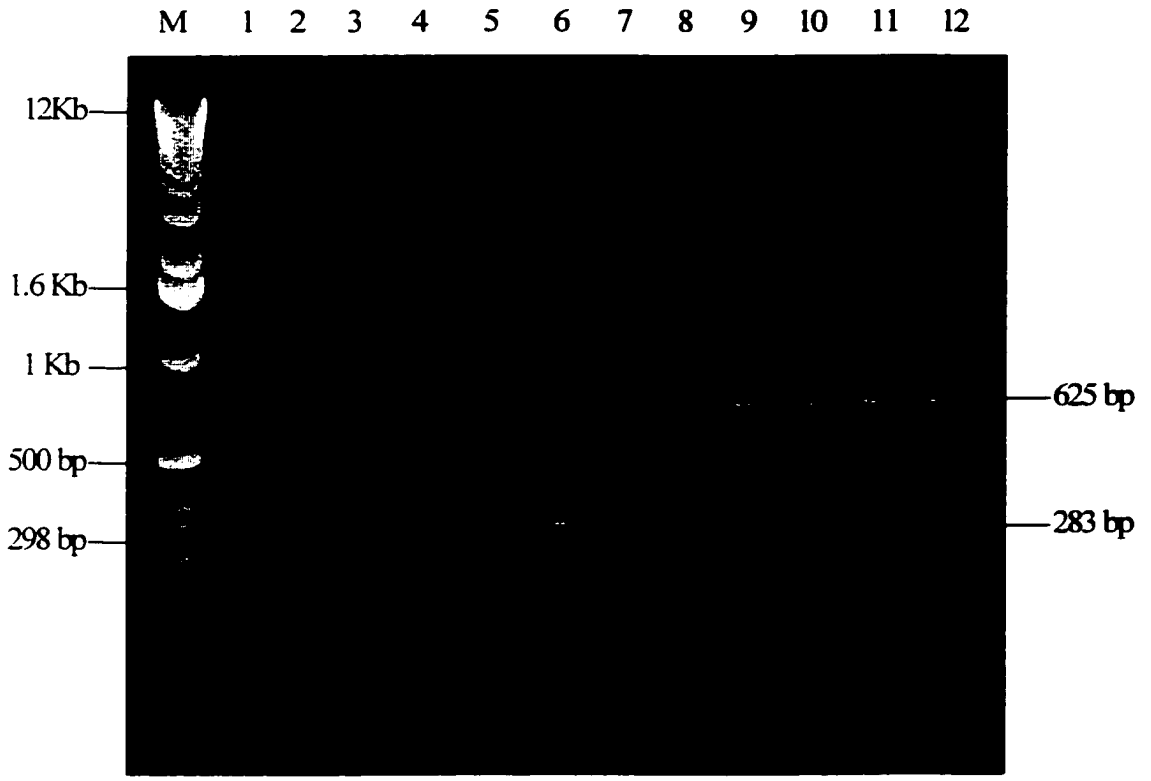


Figure 16. RT-PCR of ENV mRNA isolated from COS-1 cells transfected with the plasmids CMV/B_RT3.6 (lane 9), CMV/B_RT3.12 (lane 10), CMV/B_RT3.15 (lane 11) and CMV/B_RT1.4 (lane 12); negative controls (mRNA plus Taq polymerase) (lanes 2-4) and amplification of β-actin (283 bp) from the respective cDNA (lanes 5-8).

Expression of rgp120 by plasmid constructs in COS-1 cells. To further analyze the efficiency of the plasmid constructs, COS-1 cells were transfected with 2 μg of the respective DNA. The plasmid constructs CMV/B_RT3.6, CMV/B_RT3.12, and CMV/B_RT1. 15 and CMV/B_RT1.4 were transfected into COS-1 cells. Control cells were transfected with CMVint-BL without ENV. Lysates of the cells were tested in ELISA for

the presence of rgp120 peptides using pooled hyperimmune human sera from Ugandan donors. The selected test sera were previously shown to cross-react with the PND peptides from RT1 and RT3 (121, Appendix 3). Serum antibodies detected rgp120 in the lysates of cells transfected with each plasmid. Extracts from cells that were transfected with a plasmid CMV/BRT1.4 showed the least reactivity with the test sera (Figure 17).

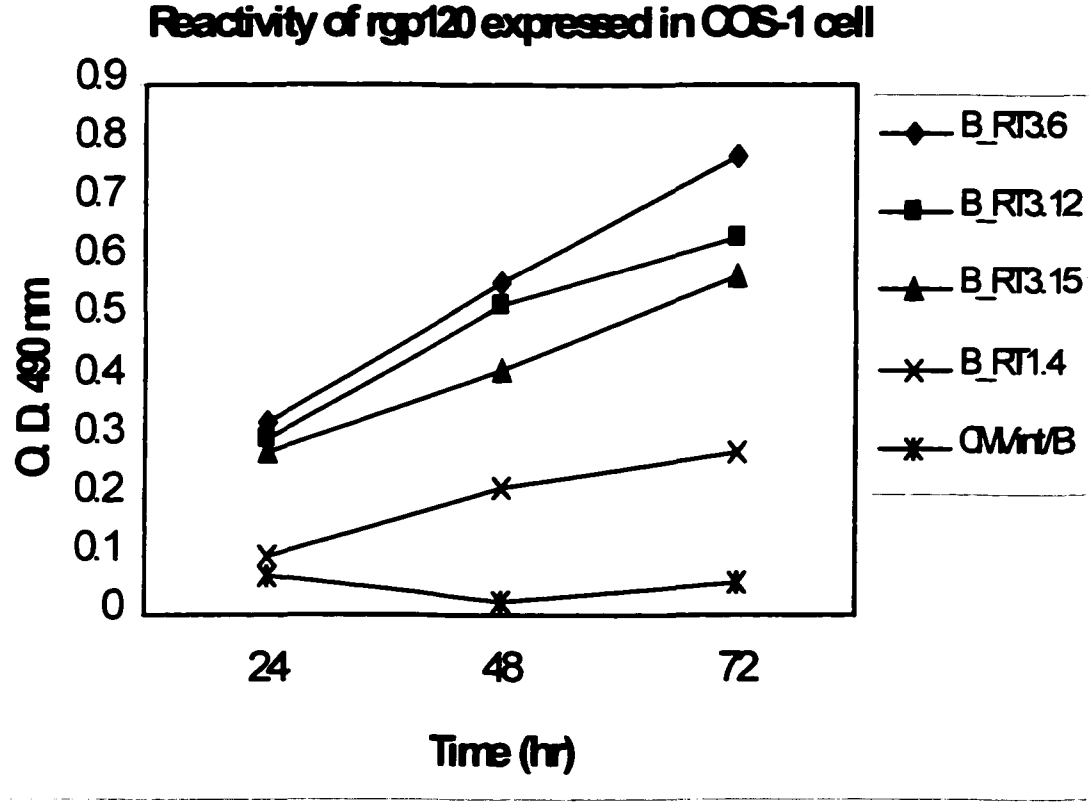


Figure 17. ELISA reactivity of rgp120 peptides expressed in transfected COS-1 cells. Transfections were conducted with the following plasmids: CMV/B_RT3.6 (◆), CMV/B_RT3.12 (●), CMV/B_RT3.15 (▲) and CMV/B_RT1.4 (■) and control cells were transfected with CMVint-BL without ENV (✱). Background reactivity of normal human serum antibodies with the cell extracts was subtracted from experimental values.

Induction of antibody response to rgp120 by plasmid-mediated immunization. To assess the relative immunogenic potential of rgp120, the plasmids CMV/B_RT3.6, CMV/C_BRT3.12, CMV/B_RT3.15 and CMV/B_RT1.4, were inoculated into the quadriceps muscle of BALB/c mice. Inoculation with the BRT3 sibling clones elicited antibodies that reacted with the homologous V3 peptide (Figure 18A and Figure 18B). These data demonstrate the utility of this approach to drive immunization against antigens from a single transcript. The present study also expands on previous work (183, 184, 195, 196, 198-204) by clearly showing that immunization with the ENV gene can generate antibody against HIV-1. Inoculation with CMV/B_RT1.4 generated near-background levels of antibody response in these studies (Figure 18C). Sera from mice inoculated with B_RT3.12 and B_RT3.15 gp120 constructs were also found to cross-react with heterologous V3 loop peptides of C_UG045 and A_UG06c (Figure 19). This pattern of cross-reactivity of the antibodies was not affected by the maturation of the immune response when tested at 6 months (Figure19B and 19D). These findings support the contention that DNA inoculation is capable of inducing conformationally relevant antigens, which may elicit cross-reactive antibody responses. The disparities noted between RT1 and RT3 with human test sera was also noted with the gp120 constructs used in DNA immunization. These results independently illustrate that sequence variation at the cap of the V3 loop can affect immunogenicity of HIV-1 primary isolates. Furthermore, this technology has relevance for the development of safe and efficacious vaccines against HIV because it provides for the relevant antigen production in vivo without the use of infectious agents.

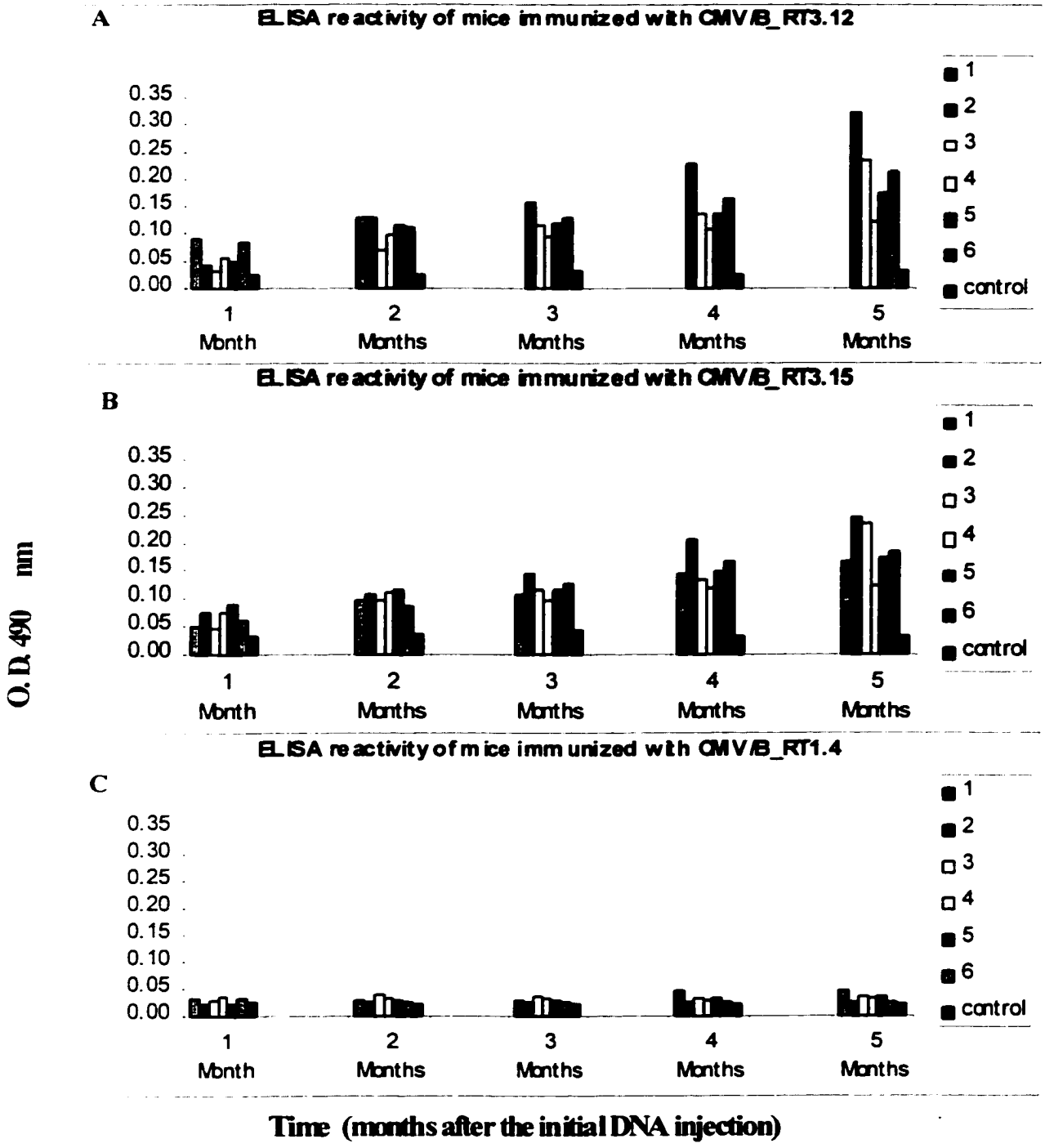


Figure 18. Relative immunogenicities of the plasmid constructs containing ENV from RT1 and RT3 sibling clones. Groups of six BALB/c mice were immunized with the plasmid CMV/B_RT3.12, CMV/B_RT3.15 or CMV/B_RT1.4. The mice were bled, and homologous V3 loop peptide was used to quantify antibody reactivity using ELISA. The mean of duplicate assays is shown.

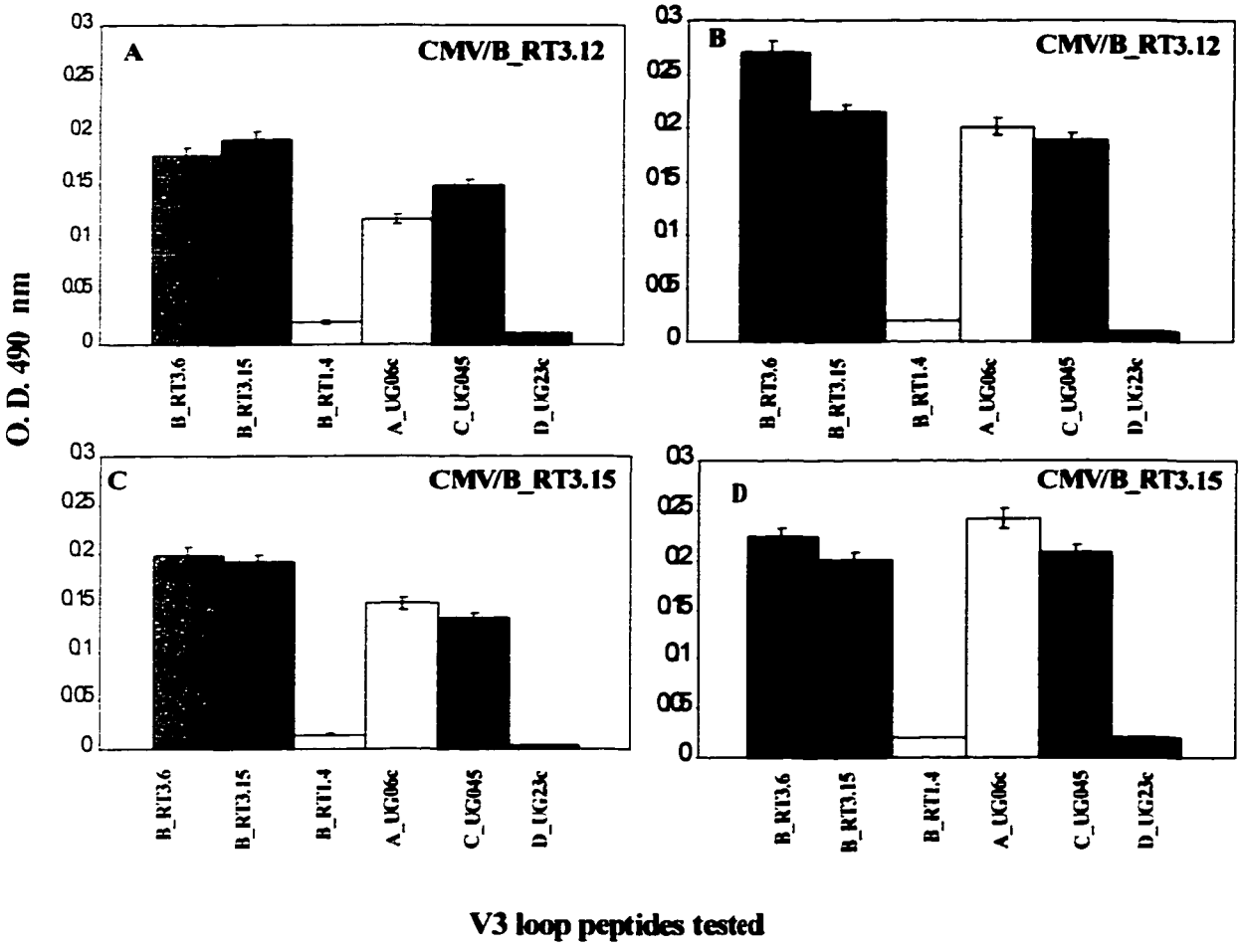


Figure 19. Cross-reactivity of sera generated by immunization with ENV plasmids: CMV/B_RT3.12 [4 months (panel A) 6 months (panel B)], or CMV/B_RT3.15. [4 months (panel C) 6 months (panel D)]. The data show the mean ±S. D. of 3 mice from for each group. Data shown are representative of five individual experiments.

In all the immunization experiments conducted it was surprising that the clones of BRT1.4 was never able to generate antibody response. These results suggest that the rgp120 encoded by B_RT1.4 may be defective in some way. Although all the plasmids were confirmed to transcribe the respective mRNA in vivo (Figure 20), it is possible that the synthetic peptides (V3 loop) tested in this experiment do not bind antibody that may have been produced. Secondly, specific structural conformation appears to play an important role in the recognition of antigens by antibodies (205, 206). A β-turn conformation in a surface-exposed region of a protein is often associated with a high probability of antibody recognition (121, 207). The amino acid residues comprising the V3 loop of RT1 and RT3 were analyzed using the BETATURN programs to determine the effect of secondary conformation on antigenicity (207, 208). As previously reported (121) my results show that the gp120 encoded in B_RT1.4 had a low probability of β-turn in the residues, GPWG, of the V3 loop (Figure 21). In contrast, the tetrapeptide GPGR (seen in RT3 sibling clones) showed a high probability of assuming a β-turn conformation. The absence of this conformation could contribute to the lack of antigenicity of the V3 loop encoded by the RT1 isolate.

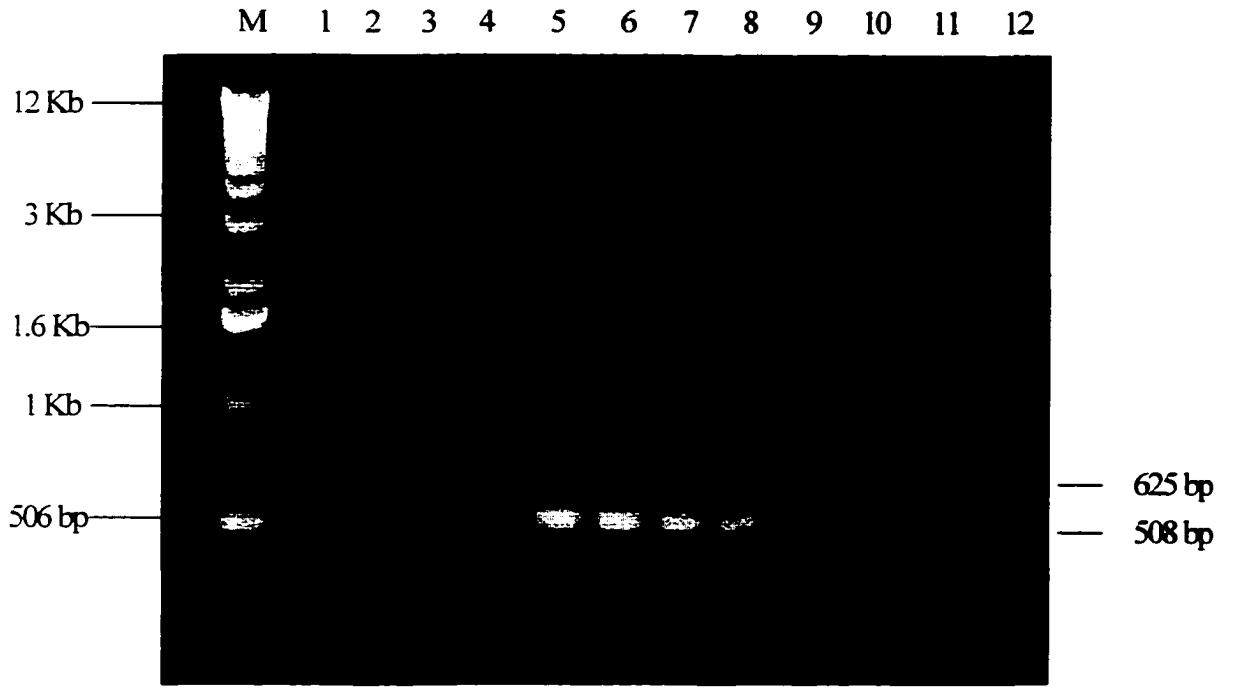


Figure 20. Transcription of ENV mRNA in quadriceps muscle of mice transfected with CMV/B_RT3.6 (lane 9), CMV/B_RT3.12 (lane 10), CMV/B_RT3.15 (lane 11) and CMV/B_RT1.4 (lanes 12) detected by RT-PCR. Transcripts were detected 7 days post-immunization. Lanes 1-4 (no reverse Transcriptase added to RT-PCR reaction); RT-PCR of β-actin (no ENV primers, lanes 5-8). DNA ladder (M).

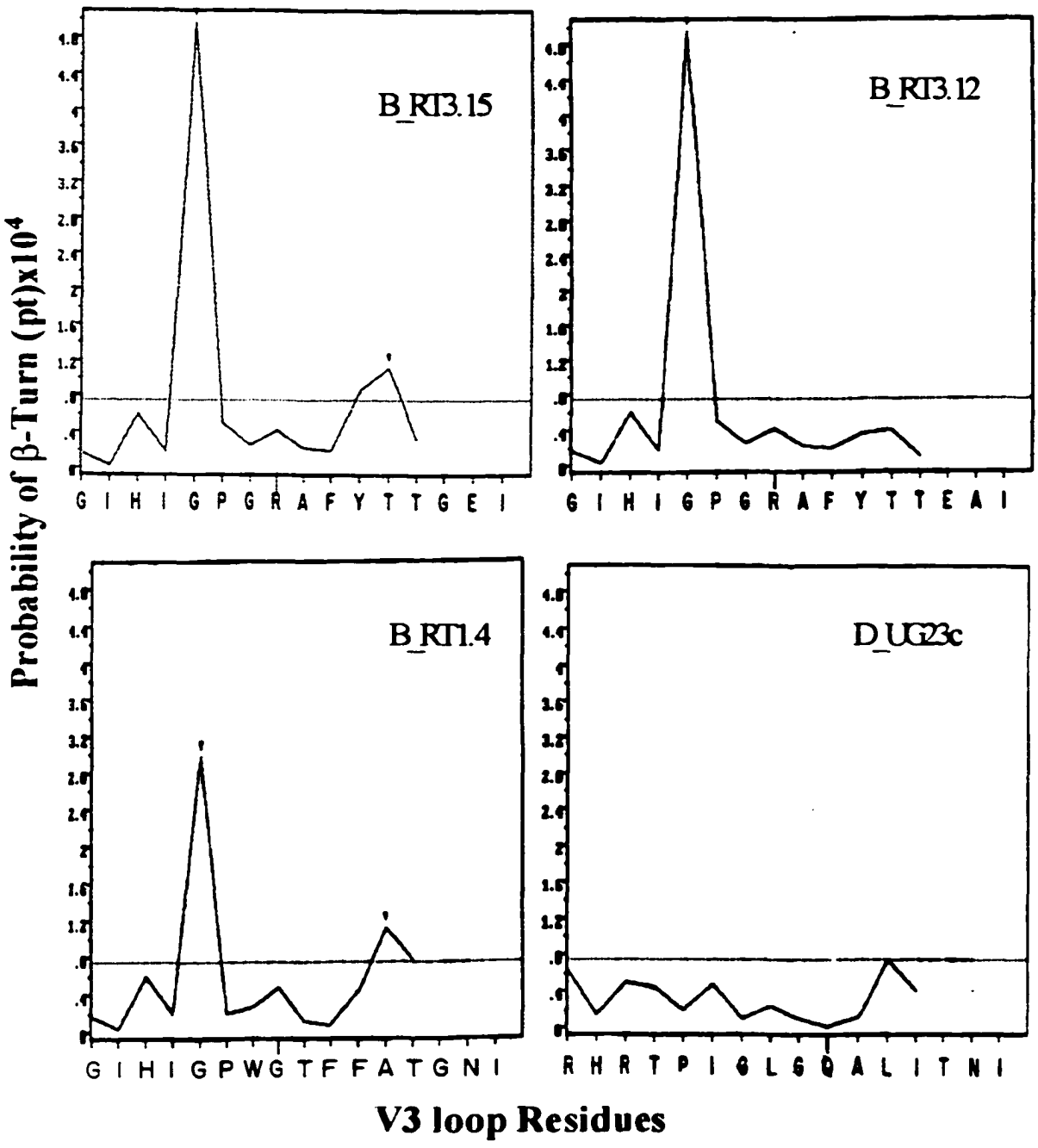


Figure 21. BETATURN analysis of B-RT3.15, B_RT3.12, B_RT1.4, and D_UG23c. β-Turn analysis. The program BETATURN uses the Chou and Fasman algorithm to predict tetrapeptide sequences which express the antigenic β-turn in protein secondary structure (208). The application of BETATURN for the determination of the antigenic regions in gp120 has been described (208).

Induction of cell-mediated immune response by plasmid constructs. Splenocytes from plasmid-inoculated mice were tested for their ability to proliferate in response to in vitro stimulation with the homologous V3 peptides. Antigen- specific proliferative responses were noted for the cells of mice immunized with B_RT3.12 and CMVB_RT3.15 when tested (Figure 22). In contrast, no response was observed for lymphocytes of mice immunized with CMV/B_RT1.4. These results highlight the impact of genetic variation in the V3 loop on its ability to elicit immune responses.

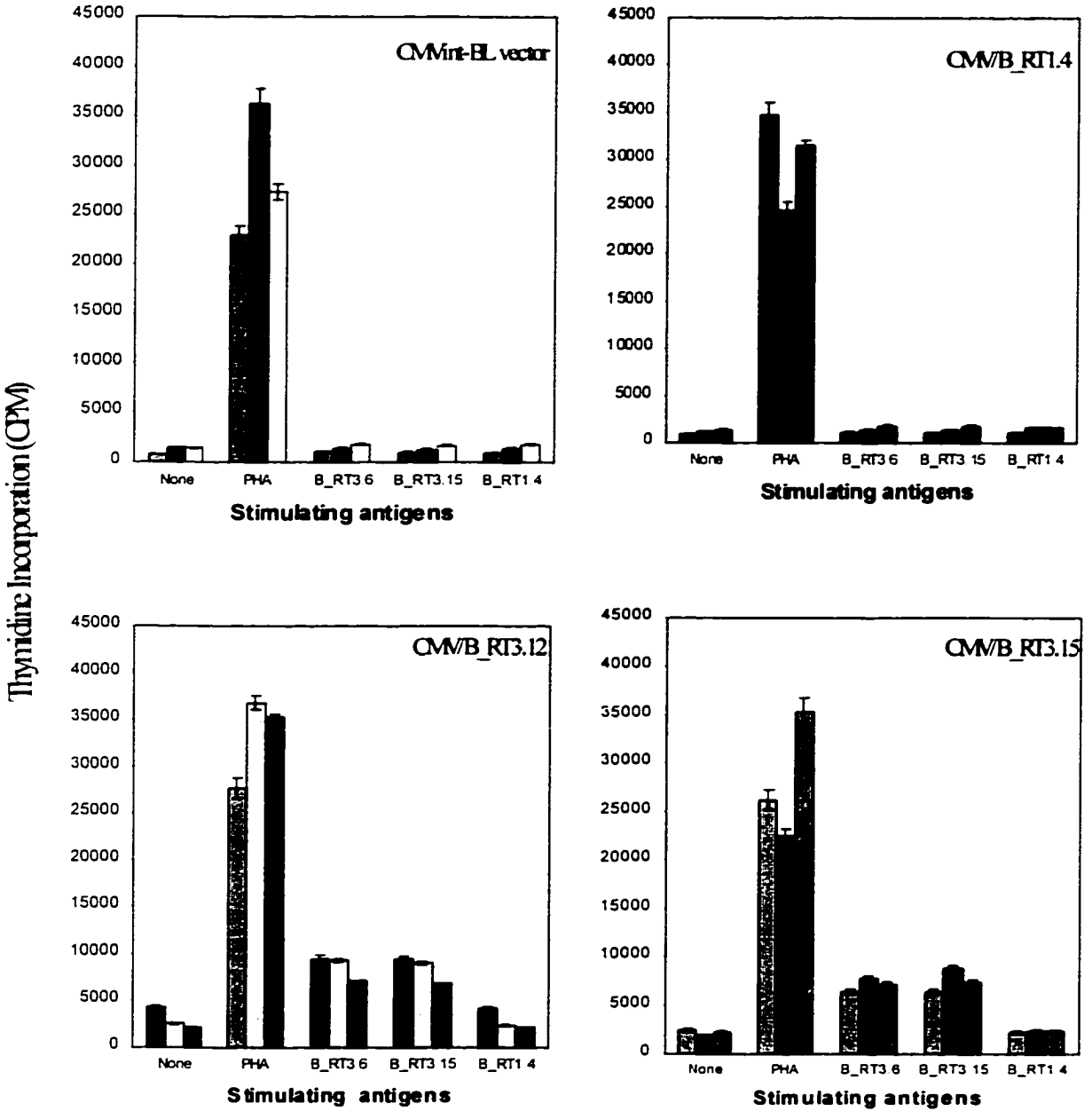


Figure 22. Incorporation of [^{3}H]Thymidine (CPM) in spleen cells of mice inoculated with the plasmids CMV/B_RT3.12, CMV/B_RT3.15, CMV/B_RT1.14 or CMVint_BL vector. Splenocytes were re-stimulated in vitro with peptides after the 6th boost. Error bar is shown for triplicate assays conducted on splenocytes from three individual mice. The results shown are representative of 3 separate experiments.

Induction of cytokine secretion. The above results collectively established that in vivo DNA immunization can induce CMI and antibody responses. In order to further evaluate the effectiveness of the immune responses, I also performed cytokine secretion assay. Previous studies have shown that PBLs from HIV-1 exposed individuals who tested negative for the virus released IL-2 when stimulated with a V3 peptide (209-212). IL-2 is a TH-1 type cytokine and there is considerable evidence that the TH1 type response plays an important role in HIV-1 protection (209-212).

To test the ability of the ENV plasmid clones to induce IL-2 production, the immune mouse lymphocytes were cultured in triplicate in 96-well microculture plates for three days. IL-2 was assayed in stimulated cell supernatants by using a commercial assay kit. Figure 23 shows that splenocytes from mice inoculated with CMV/B_RT3.12, CMV/B_RT3.15 and CMV/B_RT1.4 released IL-2. However, inoculation with CMV/B_RT1.4 induced lower levels of IL-2 than immunization with CMV/B_RT3.12 or CMV/B_RT3.15.

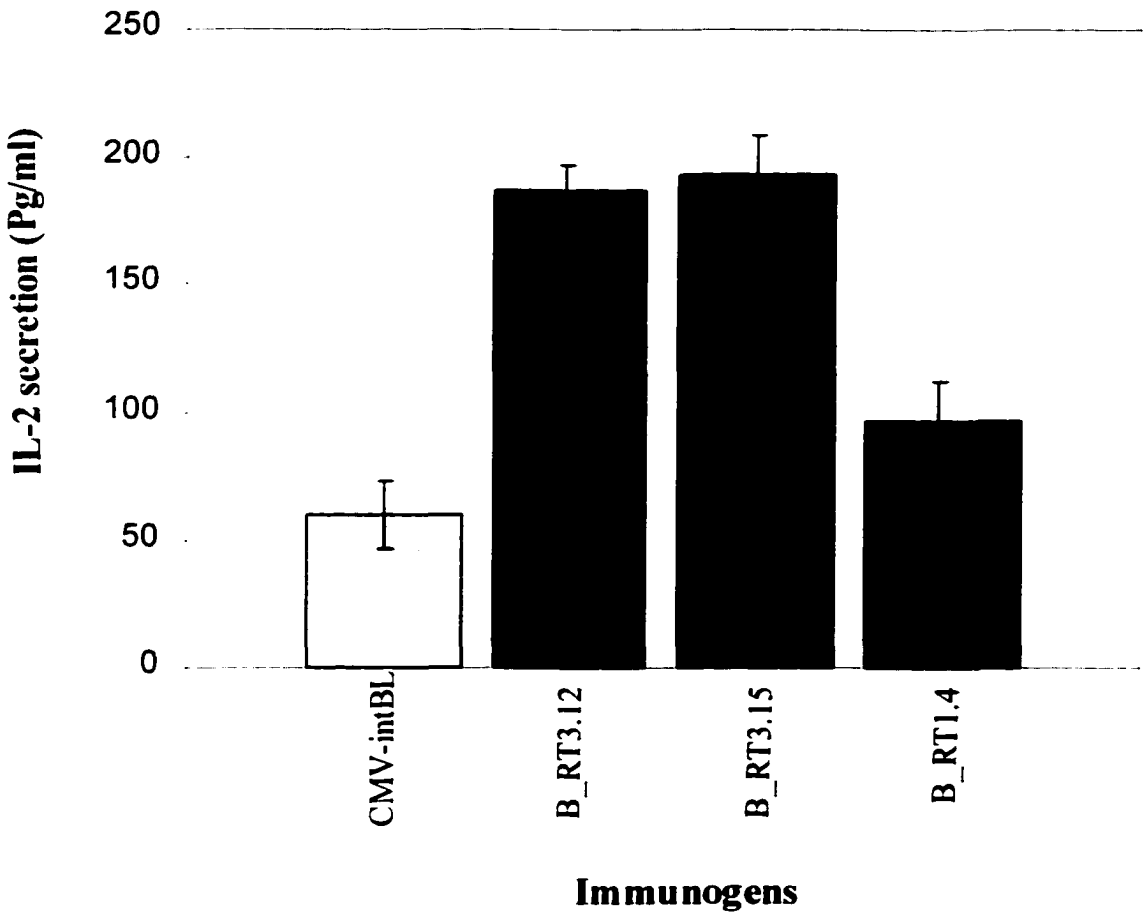


Figure 23. Splenocytes from CMV/B_RT3.12, CMV/B_RT3.15 and CMV/B_RT1.4 immunized mice released IL-2. The background values for IL-2 (75 pg/ml) secretion from non-immunized mice were subtracted from all the results. The data show the mean ±S. D. of 3 mice for each group.

Variation in the seroreactivity of MAPs comprising of the V3 loop from BRT1 and BRT3 clones. In the data presented so far, B_RT1.4 V3 linear peptide displayed background levels of ELISA reactivity with sera from HIV-1 infected donors. Additionally, inoculations with CMV/B_RT1.4 expression plasmid fail to stimulate detectable CMI responses. These results are not surprising because the significance of antibodies specific for linear sequences such as the V3 loop has been questioned as a result of experiments showing that the majority

of neutralizing antibodies in HIV-1 infected patients recognize conformation-dependent or discontinuous epitopes (11). If so, these findings would limit the neutralizing potential of vaccines based on linear sequences. Accordingly, the possibility exists that the V3 loop of B_RT1.4 may still react with antibody when presented in a different molecular conformation. Sera from eleven HIV-1 infected NY patients (and 1 normal control) were tested for reactivity against MAPs and linear forms of the V3 loop peptides from BRT1 and BRT3 clones (Figure 24). All the serum samples reacted significantly with the MAPs corresponding to the V3 loop sequences in contrast to the control sample. The antibody titers against the B_RT1.4 V3 loop MAPs were much lower in comparison to V3 loop MAPs from RT3 sibling clones. In addition, a significant difference was seen between the reactivities of most of the sera with the V3 loop peptides in the MAP and the linear forms (Figure 24). The difference in the antibody reactivity observed from these forms of peptides may be attributed to conformational characteristics of the test peptides (183, 184). In addition, a lysine core bearing four reactive NH_2 termini served as the carrier onto which four V3 sequences were attached by solid phase synthesis of MAP. This MAP matrix with a four-fold molar excess of the peptide core could account for the varying antibody reactivity noted for the test peptides.

Figure 24B shows that there was a sight but significant difference between the MAP and linear forms of the V3 loop derived from B_RT1.4. This results suggest that MAP presents the V3 epitope of B_RT1.4 in a conformation that reacts better with antibodies and raises the possibility that this conformation could affect the antigenicity of B_RT1.4 in a MAP mediated immunization protocol. The immunogenic properties of MAPs comprising of the V3 loop from B_RT1.4, B_RT3.6 and B_RT3.15 were tested in ELISA, proliferation and cytokine secretion assays.

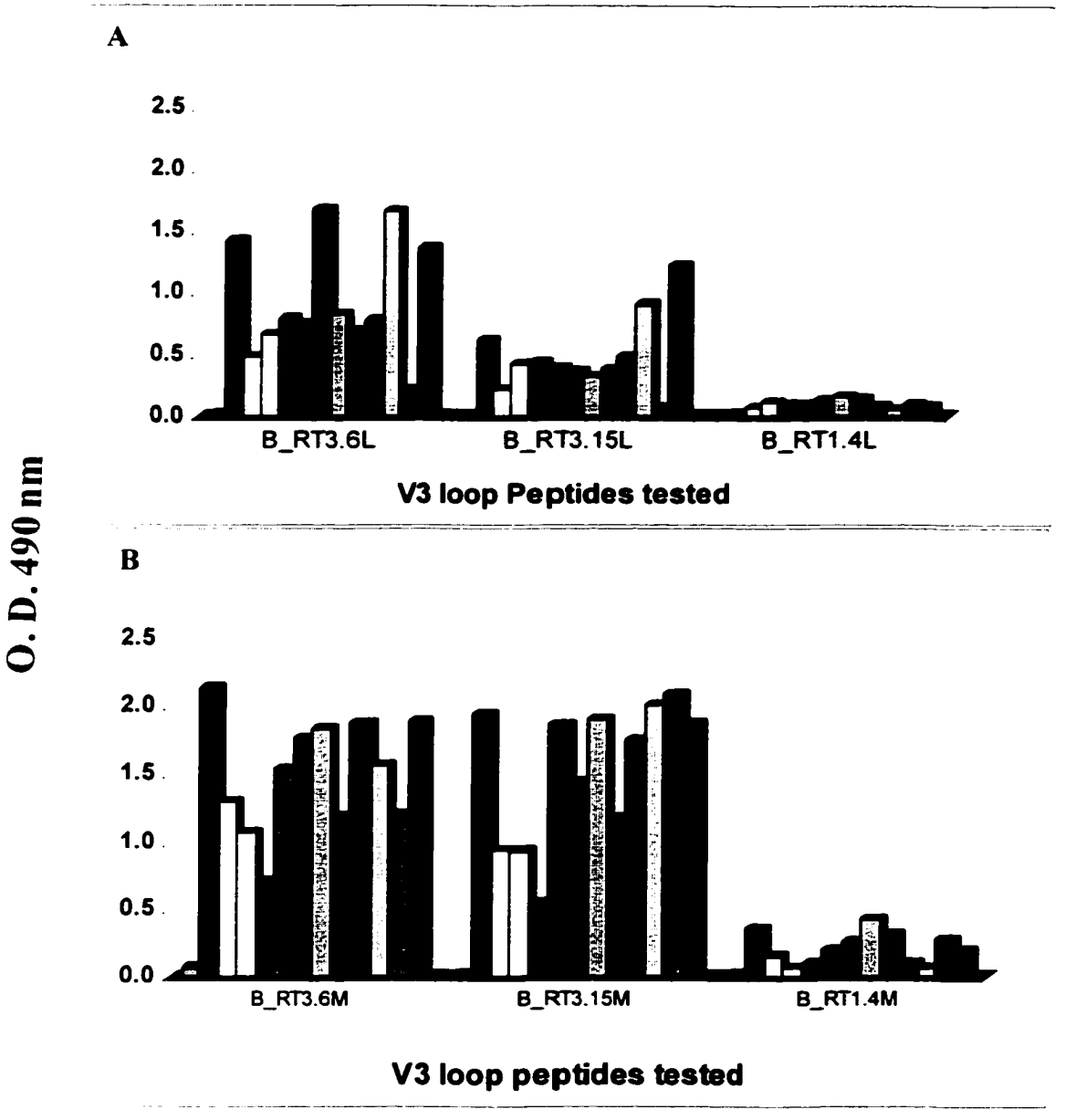


Figure 24. Reactivity of serum samples from asymptomatic New York donors with synthetic linear peptides (A) and MAPs (B) comprising the predicted E2[V3] epitopes from B-RT3.6, B_RT3.15, and B_RT1.4. The mean of duplicate assays is shown. Abbreviations: B_RT1.4M and B_RT1.4L indicates the MAPs and linear peptide, respectively, consisting of the V3 loop from the RT1 isolate; B_RT3.6M, B_RT3.6L, B_RT3.15M and B_RT3.15L indicates the MAPs and linear peptide, respectively, consisting of the V3 loop from the RT3 isolate.

Induction of antibody response by MAPs-mediated immunization. MAPs comprising of the V3 peptides of B_RT3.6, B_RT3.15 and B_RT1.4, were inoculated into the quadriceps muscle of BALB/c mice. Inoculation with MAPs elicited very high levels of antibodies that reacted with the homologous V3 PND peptide of B_RT3.6 or B_RT3.15 (Figure 25A and 25B). Antiserum from mice inoculated with B_RT3.6, and B_RT3.15 MAPs were also found to cross—react with heterogeneous V3 loop peptides of C_UG045 and A_UG06c (Figure 25). These findings support previous studies showing that MAPs inoculation is capable of inducing conformationally relevant antigens, which elicit cross-reactive antibodies (213-219). In sharp contrast, inoculation with V3 loop MAPs from BRT1.4 generated low levels of antibody response (Figure 25C). These data apparently highlight a clear impact of primary variation on the immunogenity of the gp120.

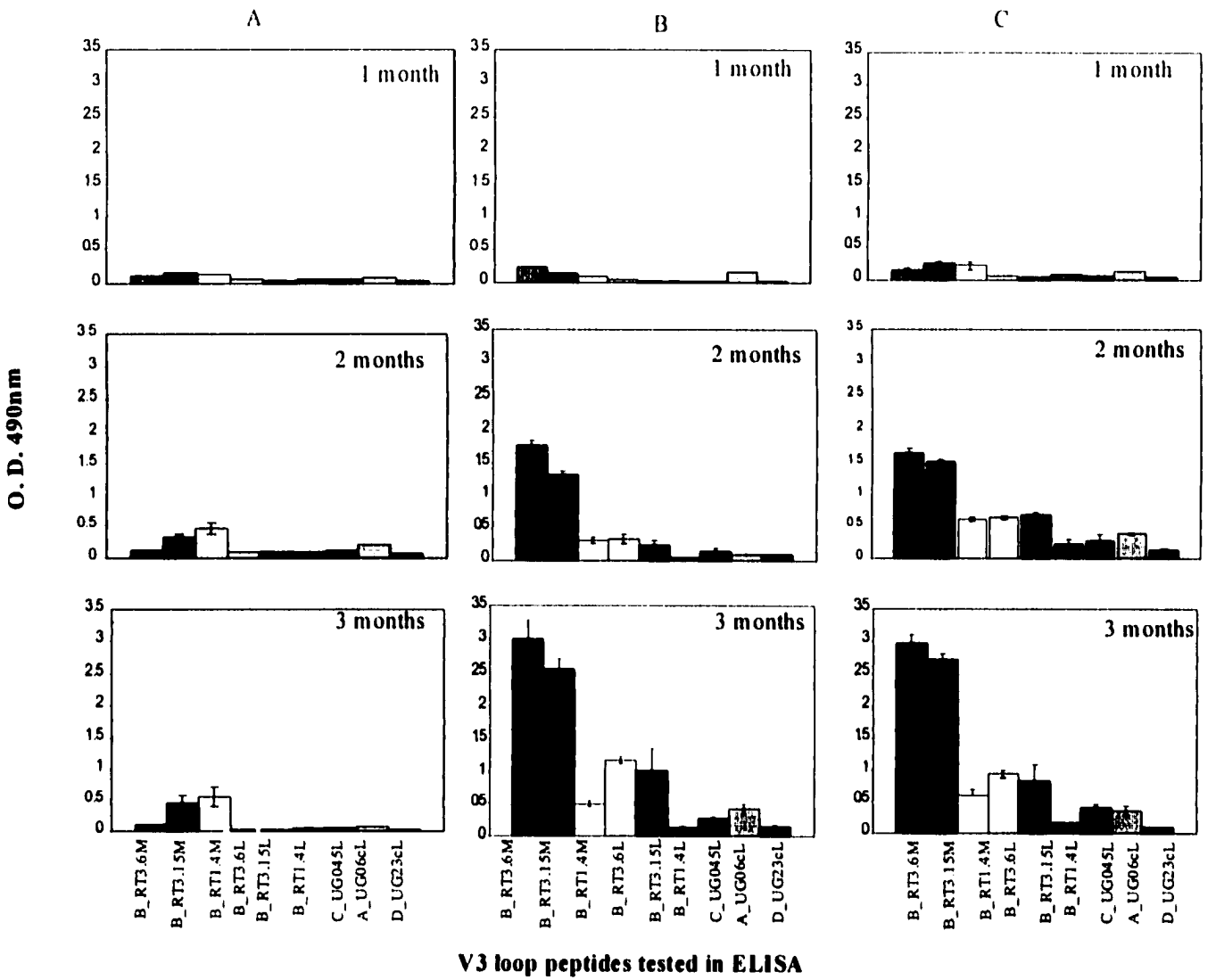


Figure 25: MAPs consisting of the PND of B_RT1.4 (panel A), B_RT3.6 (panel B) and B_RT3.15 (panel C) were inoculated into the quadriceps muscle of BALB/c mice. Inoculation with MAPs from B_RT3.6, and B_RT3.15 elicited antibodies that reacted with the homologous and heterologous V3 PND peptides. The data show the mean ±S. D. of 3 mice from each group tested. Abbreviations: B_RT1.4M and B_RT1.4L indicates the MAPs and linear peptide, respectively, consisting of the V3 loop from the RT1 isolate. Similarly, B_RT3.6M, B_RT3.6L, B_RT3.15M and B_RT3.15L indicates the MAPs and linear peptide, respectively, consisting of the V3 loop from the RT3 isolate.

Induction of CMI responses by MAPs immunization. The results presented above support the view that inoculation with MAPs is capable of eliciting immune responses. For this reason T cell proliferation assay was performed on spleen cells of mice that had been immunized with MAPs consisting of the V3 loop from B_RT1.4, B_RT3.6 and B_RT3.15. Splenocytes from MAPs inoculated mice were compared to adjuvant inoculated controls for their ability to proliferate in response to specific in-vivo re-stimulation with homologous V3 loop linear peptides. Antigen- specific proliferative responses were noted for mice inoculated with MAPs consisting of the V3 loop from B_RT3.6 and B_RT3.15 (Figure 26) but the responses of mice immunized with B_RT1.4 MAP were low and indistinguishable from the response of mice immunized with the adjuvant alone. These results ate similar to those obtained by DNA immunization and independently demonstrate the impact of primary sequence variation on the immune responses to gp120.

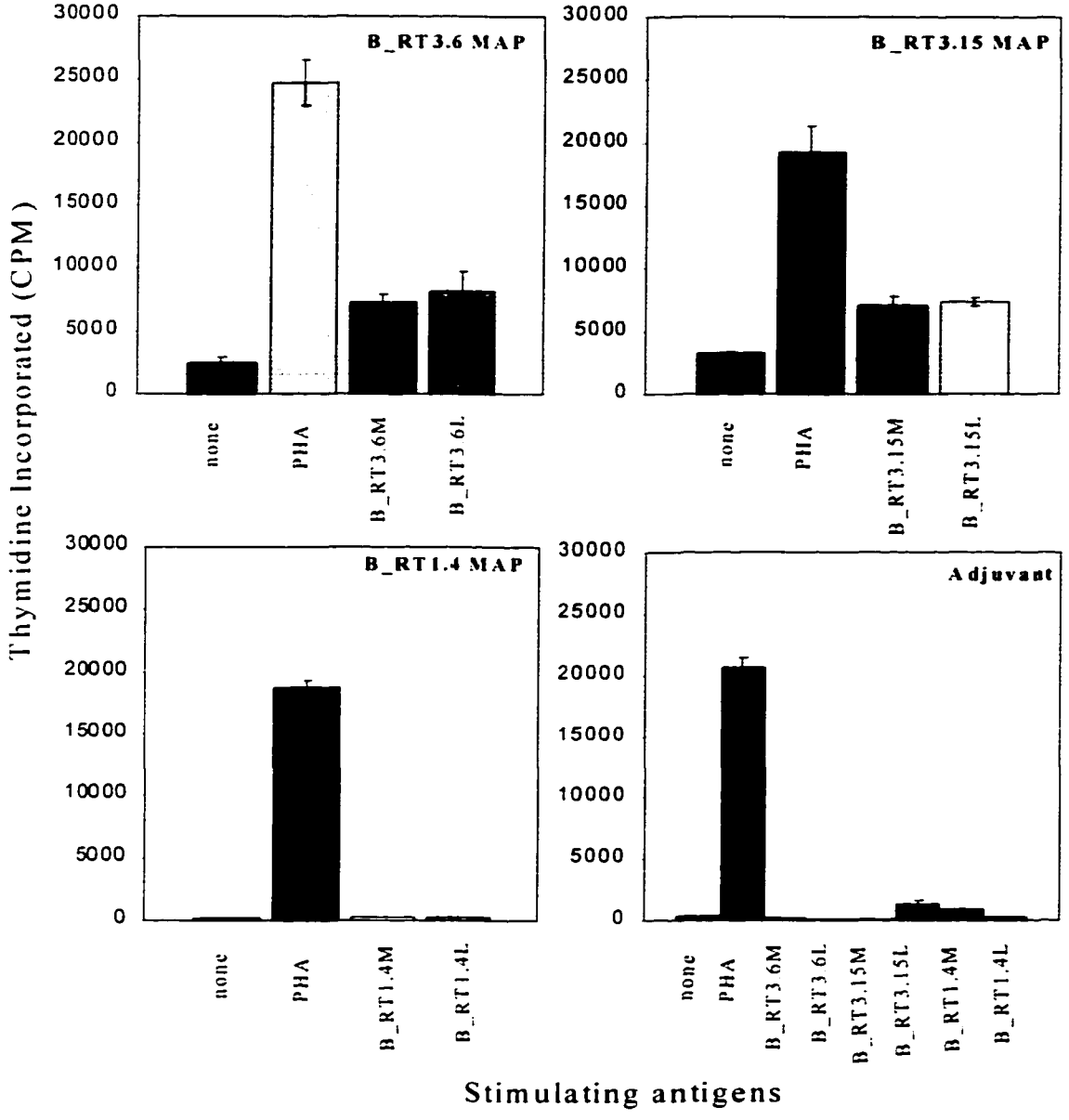


Figure 26. Incorporation of [^{3}H]Thymidine (CPM) in spleen cells of mice inoculated with the MAPs consisting of the V3 loop from B_RT3.6, B_RT3.15, or B_RT1.14. Splenocytes were re-stimulated in vitro with peptides after the 3rd boost. Each value is the mean and standard deviation of triplicate assays. Data shown are representative of five individual experiments. Abbreviations: B_RT1.4M and B_RT1.4L indicates the MAPs and linear peptide, respectively, consisting of the V3 loop from the RT1 isolate. Similarly, B_RT3.6M, B_RT3.6L, B_RT3.15M and B_RT3.15L indicates the MAPs and linear peptide, respectively, consisting of the V3 loop from the RT3 isolate.

IL-2 secretion by cultured cells. The immunogenic potential of B_RT3.6, B_RT3.15 and B_RT1.4 was further tested in a cytokine assay. To test the ability of mice immunized with MAPs to induce IL-2 production, lymphocytes were cultured in triplicate in 96-well microculture plates for three days. Spontaneous release of IL-2 was determined in stimulated cell supernatants with a commercial assay kit. Figure 27 shows that splenocytes from mice immunized with the V3 loop MAPs of B_RT3.6, B_RT3.15 and B_RT1.4, released IL-2. Immunization with B_RT1.4 MAP stimulated lower levels of the lymphokine.

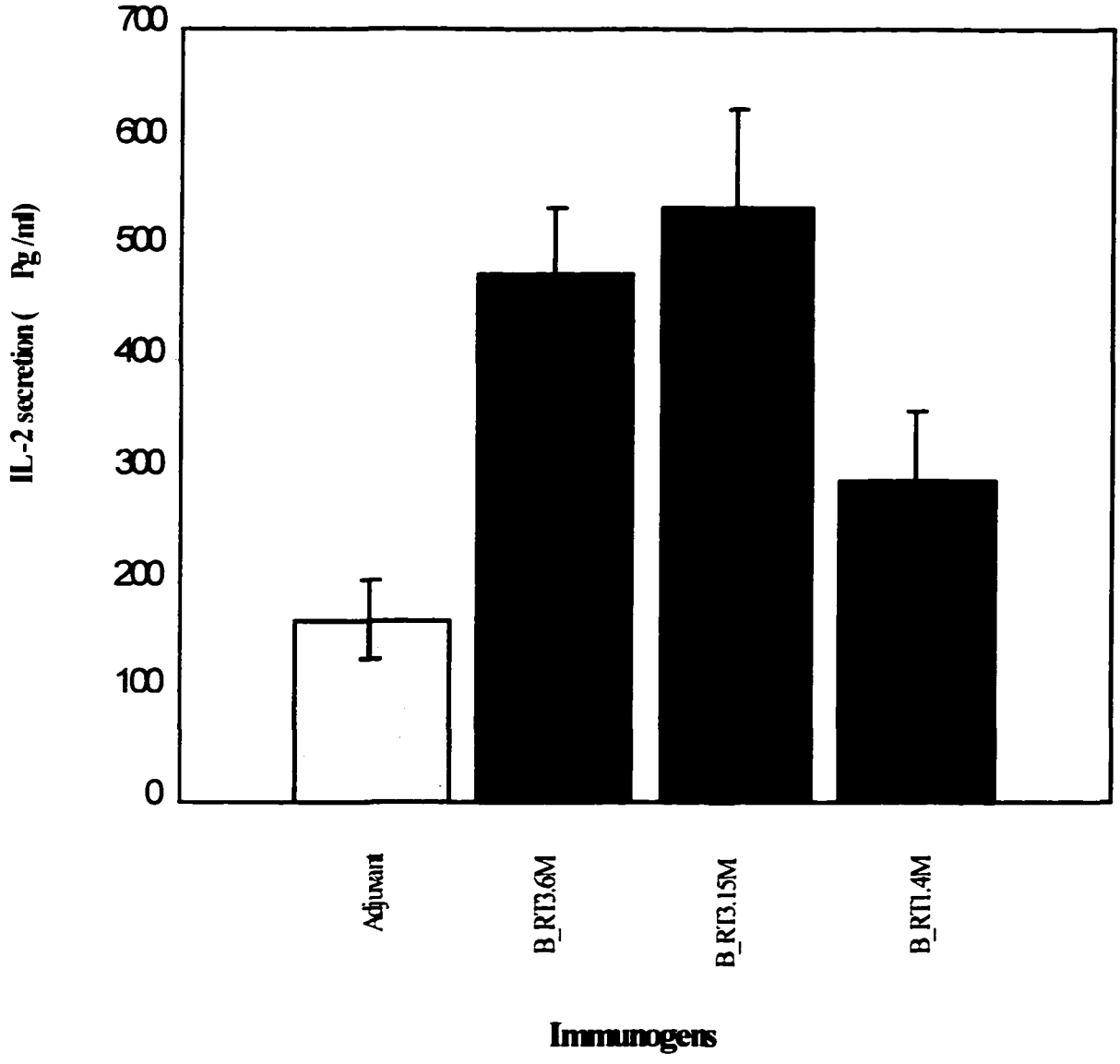


Figure 27. IL-2 levels in 72 hr culture supernatant of splenocytes from mice immunized with the V3 loop MAPs of B_RT3.6, B_RT3.15 and B_RT1.4. The background values for IL-2 (75 pg/ml) secreted by non-immunized mice were subtracted from all the results. The data show the mean ±S. D. of 3 mice for each group tested in this study. Abbreviations: B_RT1.4M indicates the V3 loop MAPs from the RT1 isolate. Similarly B_RT3.6M, and B_RT3.15M indicate the MAPs consisting of the V3 loop from the RT3 sibling clones.

DISCUSSION

In other retroviral systems, the envelope glycoprotein has been shown to be the target of neutralizing antibodies or cell-mediated immunity (reviewed in 11). Likewise, recombinant gp120 expressed in mammalian and bacterial cells have been shown to elicit the production of neutralizing antibodies in animal models (134, 135, 137). The genetic diversity of HIV-1 is a major concern thought to impact on the neutralizing efficacy of gp120 candidate immunogens. Variation in the region of the provirus encoding gp120 has been shown to directly influence the antigenic HIV-1 phenotype. In particular the third hypervariable domain (V3) is implicated in a number of biological properties of the virus (reviewed in 11). Phylogenetic tree analysis of the nucleotide sequences that encode the C2-V5 region of gp120 has shown that both HIV-1 isolates, RT1 and RT3, derived from an asymptomatic and an AIDS subject, respectively, segregate with the subgroup "B" viruses (Figure 11). Marked variations were noted in the nucleotide and predicted amino acid sequences that comprise the V3 loop, and other antigenic sites of gp120 in the two isolates, RT1 and RT3. The sibling clones, B_RT3.10, B_RT3.11, B_RT3.12, and B_RT3.15 derived from RT3 exhibited the North American consensus peptide GPGRAF in the antibody binding site at the apex of the V3 loop. However, the clones B_RT1.4, B_RT1.15, B_RT1.17 and B_RT1.21 derived from a newly infected (asymptomatic) donor encoded the novel hexapeptide GPWGTF at this location (Figure 12).

Computer-assisted analysis of the C2-C5 domains of gp120 region of RT1, RT3 derived clones, and reference isolates MN and D_UG23c, was undertaken to assess the effect of primary sequence variation on the distribution of the antigenic sites (Figure 12). These data showed that despite extensive inter-isolate primary sequence diversity, many of the predicted epitopes were conserved in their relative location on gp120. However, the epitope (E2)[V3] expressed in the clones of the isolate RT3 are clearly unique. This epitope is

remarkably extensive in comparison to the analogous site so far documented for any HIV-1 isolate including MN (Figure 12). This region of the V3 loop has been extensively studied and has been shown to generate high titers of neutralizing antibodies and CMI response which are subtype-specific (157, 158). It is uncertain whether the extensive profile of antigenicity of the V3 loop in the sibling clones derived from RT3 would enhance the immunogenity of gp120 in vivo.

To investigate this possibility, the levels of antibody binding by synthetic peptides comprising the epitope E2[V3] of B_RT3.6, B_RT3.15, and B_RT1.4 were compared. Inter-clade and intra-clade variation in ELISA reactivity was noted for these peptides (Figure 14). Previous investigations (97-102) have shown that HIV-1 consists of a large number of genotypes (quasispecies) as shown by variation in the GAG and in the variable regions of ENV. However, the role of these multiple variants in the pathogenesis of AIDS is unclear. The present data have shown diversity in the antigenic properties and seroreactivity of these inter-subject and intra-subject quasispecies. The most extensive divergence among the RT3 sibling clones appeared to be due to amino acid substitutions downstream of the V3 apex (Figure 12). These results confirm the accumulating evidence that there are high levels of genetic variation among HIV-1 isolates in infected individuals (219-223). Efforts to develop effective HIV vaccines have been hampered by the lack of a systematic approach to study the extraordinary diversity of the HIV-1 quasispecies.

One of the factors that determine the immunogenicity and neutralization of HIV-1 is the structural conformation of gp120 that exposes potential antigenic sites to antibody recognition (11, 121). No studies have been conducted to date to adequately determine the effect of primary sequence variation on the conformational structure of gp120 (68, 206). Nevertheless, it is known that a number of factors, such as hydrophilicity, β-turns, and flexibility, contribute to the antigenicity of proteins in general. These variables may well contribute to the different levels of seroreactivity of the clones examined in this study.

The use of hyper-immune human sera for a comparison of divergent gp120 immunogens is flawed because, the time of infection is unknown and the subtype of the virus is often unknown. A more definitive approach is to generate and use antibodies developed by immunization with rgp120 of defined "origin" and specificity. The recent strategy of genetic immunization employs a non-integrating nucleic acid (DNA or RNA) construct that encodes a predetermined immunogen (183, 184, 187, 188). In theory, direct genetic immunization could mimic some aspects of attenuated vaccines in that synthesis of the specific proteins could be accomplished in the host and thus be subject to immune recognition. However, genetic immunization is dependent upon the injection of the nucleic acid construct directly into a host target tissue which then expresses the desired protein (187-204). This approach has been successfully used to generate protective immunity against the influenza virus in mice and chickens (188), and bovine herpesvirus in mice and cattle (190) and choriomeningitis virus in mice (191). In addition, a number of genetic immunization studies using DNA encoding viral HIV-1 antigen have demonstrated antigen-specific cellular and humoral immune responses (183,184, 195, 196, 198-204) and phase 1 trials have been conducted to assess the safety and immunogenicity of HIV-1 ENV DNA in human subjects (224-227). Encouraging evidence of the use of DNA vaccines was described for HIV-1 DNA vaccine containing env, rev, and gag/pol: immunization increased HIV-specific cellular immune responses (224-227).

To further investigate the effect of genetic variation on immunogenicity, CMVint-BL plasmids that contain and express a 625 bp fragment of the ENV gene was constructed. ELISA and RT-PCR results (Figure 16, Figure 17) have shown that the constructs contain and express the ENV gene. The proviral DNA clones were then used in a plasmid-mediated immunization protocol to define the immunogenity and antigenic specificity of the antibodies elicited by the respective rgp120 peptides. Antibody responses to the homologous V3 peptide were noted (Figure 18). These results support previous studies indicating that plasmid-mediated immunization with ENV clones elicits ELISA

reactive antibodies in mice (184, reviewed in 199, 203, 204). I had previously investigated the antigenic properties of gp120 encoded in B_RT3.6, B_RT3.15, and B_RT1.4 using hyperimmune human sera in ELISA (123). These earlier studies had shown that the epitopes presented in the sibling clones B_RT3.6 and B_RT3.15 are highly antigenic but B_RT1.4 showed negligible reactivity with the test sera. The clones derived from isolates RT1 and RT3 displayed divergent hexapeptides at the cap of the V3 loop even though both isolates clustered in clade B. The clones derived from RT1 displayed GPWGTF, while RT3 displayed the highly conserved North American hexa-peptide GPGRAF (155, 168).

Antibodies to linear V3 epitopes induced by infection are always directed against the tip of the loop (168). In the present study inoculation with these sibling clones, B_RT3.12, and B_RT3.15 was shown to induce comparable levels of antibodies while immunization with CMV/B_RT1.4 generated near background levels of antibody responses to homologous V3 loop peptides (Figure 18). These results further showed that genetic variation may influence immunogenity of the gp120 constructs. The antibodies generated by the mice inoculated with CMV/B_RT3.6 and CMV/B_RT3.15 showed cross-reactivity with heterologous peptides from clade A and clade C but not with the peptide from clade D (Figure 19). Genetic analyses of the V3 sequences have shown that most HIV-1 isolates from clade A, B, and C express the apical tetrapeptide, GPG(R/Q) at the cap of the V3 loop (97). These tetrapeptides have been observed in this position for the majority of the HIV-1 isolates (168).

The conservation of critical amino acid residues comprising the V3 loop apex may explain the serological cross-reactivity observed among the gp120 sequences derived from divergent clades (155, 168). ELISA conducted with V3 loop epitopes of B_RT3.6 and C_UG045 displayed similar trends and intensities of reactivity with serum samples obtained from African donors (122). It should be noted that although antibody cross-reactivity to the V3 loop does not necessarily predict the ability of the antibodies to broadly neutralize the virus isolates bearing the corresponding V3 regions, a number of studies have shown that

accumulating data from studies of HIV-1 infected individuals supports the hypothesis that in primary isolates of different clades and phenotypes, common antigenic structures must exist that stimulate cross-reactive or broadly reactive immune response.

An important goal of the current search for immunoprophylactic agents to control the spread of HIV-1 is the development of a safe vaccine that can assure long-term protection. In the present study lymphocyte proliferative response to the homologous PND peptide were noted for RT3 sibling clones following DNA-mediated immunization (Figure 22). In contrast to the immunogenic potential demonstrated for RT3 sibling clones, results for the analogous molecule from RT1 were negligible. The fact that the mice were immunized with B_RT3 and yet still responded to heterologous peptides in vitro suggests that this immunization procedure may prime proliferative responses to diverse isolates. IL-2 is a TH-1 type cytokine and there is considerable evidence that the TH1 type response plays an important role in protection of HIV-1 infection (146, 209-212). Splenocytes from CMV/B_RT3.12, CMV/B_RT3.15 and CMV/B_RT1.4 immunized mice released IL-2 in culture (Figure 23).

There is now abundant evidence that DNA vaccines show promise (183, 184, 187-204,) but there are still some reservations. For example, very small amounts of the antigens are sometimes produced and the plasmid could conceivably integrate into the chromosomal DNA to generate tumors (230, 231). In addition, anti-DNA antibodies might be generated. In this present study, immunization with DNA from HIV-1 isolates RT3 and RT1 gave sub-optimal or no antibody responses respectively. The relatively low titers obtained with these isolates compared with that reported for other constructs (183, 184) probably reflect the different V3 loop sequence used in each study. In an effort to enhance the immunogenicity of these PNDs, the V3 peptides were generated using the MAP method (175, 176). Synthetic peptide constructs have a number of properties that make them particularly good immunogens. For example, the immunogens can be designed to incorporate different regions from the same or different molecules (176). In addition, synthetic peptides can be used to

stimulate functional immune responses (232-236). Finally, there is no danger of residual infectivity or pathogenicity associated with the use of the whole virus or attenuated virus vaccines. However, the use of peptides as immunogens also presents some drawbacks. In particular, small linear peptides are usually poor immunogens. However, Tam et al. (176) have developed oligomeric peptides based on a lysine core.

In order to evaluate the immunogenic potential of the V3 loop sequences, the MAPs consisting of the V3 epitope from RT1 and RT3 clones (B_RT3.6, B_RT3.12, B_RT3.15 and B_RT1.4) were synthesized and inoculated into the quadriceps muscle of BALB/c mice. In these studies, successful seroconversion occurs in more than 80% of the mice immunized with the MAPs consisting of the V3 epitope from RT3 (B_RT3.6, B_RT3.12, B_RT3.15). Furthermore, repeated inoculation with MAPs from B_RT3.6, and B_RT3.15 elicited very high levels of antibodies that reacted with the homologous and heterologous V3 peptides while a considerably lower response was observed for B_RT1.4 immunized mice (Figure 25). While these findings suggest the potential usefulness of the V3 MAPs vaccine of RT3, in vivo efficacy experiments using primates are necessary to evaluate whether these titers are sufficient to block infection of HIV-1 challenge. These results further showed that genetic variation may influence immunogenity of gp120. These findings are comparable with previous data in which MAPs consisting of 8 HIV-1 IIIB V3 sequences were shown to elicit relatively high and sustained levels of HIV-1 specific neutralizing antibodies in animals without the use of a carrier protein (213, 214, 227). Recent observations suggesting that the V3 loop may be important in gp120-CD4-chemokine receptor interactions (24, 34), coupled with advantages of using a combination of peptides as a strategy for overcoming the problems of sequence diversity among HIV-1 isolates, leaves peptides with a potential role in HIV-1 vaccine development.

Interestingly, immunization with V3 MAPs produced higher amounts of antibodies than immunization with the respective plasmids. The differences observed may be due to the kind of expression vector that was used in this study. On the other hand, this variation may

be due to the way in which each method expresses the antigen in vivo for the generation of the humoral immune response. Many attempts have been made to enhance the antibody and CMI responses generated by DNA immunization (237-241). These attempts include boosting with V3 loop peptides or recombinant ENV protein (239, 240), co-administration of cytokine gene or B-chemokine receptor expression cassettes (237,241).

In addition to studying the humoral responses generated by DNA and MAP immunizations, cytokine secretion and proliferation assays were performed. IL-2 secretion and lymphocyte proliferative responses to the homologous PNDs were noted for mice immunized with MAPs from RT3 sibling clones (Figure 26 and Figure 27).

In this study I have focused on the induction of antibody and lymphocyte proliferative responses to HIV-1 antigens; however, the induction of other types of immune responses also occurs following the administration of HIV-1 DNA and peptide vaccines (23, 243-245). An increase in cytotoxic T cell activity and CTL precursors has been reported following the administration of an envelope vaccine (197-204, 225-243), and enhanced TH1 and TH2 type cytokines responses to the immunogens also can be induced (197-204).

The results presented herein together with other reports (197-204, 228-247) strongly indicate that the introduction of gp120 DNA constructs and V3 synthetic MAPs directly into muscle of animals have the capacity to induce an immune response. The response includes both the humoral and cellular arms of the immune system. The ability of genetically inoculated envelope clones to generate reactive immune responses as evaluated above may represent an important feature in vaccine design for HIV-1. Additionally, I have observed that genetic variation can impact immunogenicity of gp120 encoded in the different HIV-1 field isolates. These data also demonstrated the utility of this approach to drive immunization against multiple antigens from a single transcript. The safety and potential immunogenicity of a HIV-1 DNA-based vaccine has been demonstrated. The DNA immunogens used in these studies are from clade B. To be effective against the full antigenic spectrum of primary isolates, candidate vaccines should contain immunogens that are

representative of the whole spectrum of viral clades. There is an urgent need to identify these immunogens and to improve their immunogenic potential.

APPENDIX 1

(A). Alignment of the ENV nucleic acid sequences of the RT1 and RT3 sibling clones used to generate the phylogenetic tree. ":" character to show that a position in the alignment is perfectly conserved. "."character to show that a position in the alignment is well conserved.

```
BRT36     A------GTCTAGCAGAAGAAGAAGTAGTAATTAGATCTGCCAATTTCACGGACAA     50
BRT312    A------GTCTAGCAGAAGAAGAAGTAGTAATTAGATCGGCCAATCTCACGGACAA     50
BRT315    A------GTCTAGCAGAAGAAGAAGTAGTAATTAGATCTGCCAATCTCACGGACAA     50
BRT14     ATCCCAGTCTAGCAGAAGAAGAGGTAGTAATTAGATCTGAGAATTTCACAAACAA      55
          :      :::::::::::::::::::.:::::::::::::::::.:   ::: :::::.::::

BRT36     TGCTAAAACCATAATAGTACAGCTGAACAAATCTGTAGAAATTAATTGTACAAGA     105
BRT312    TGCTAAAACCATAATAGTACAGCTGAACAAATCTGTAAAAATTAATTGTACAAGA     105
BRT315    TGCTAAAACCATAATAGTACAGCTGACCAAATCTGTAAAAATTAATTGTACAAGA     105
BRT14     TGCTAAAATCATAATAGTACATCTGAATGAATCTGTAGAAATTAATTGTACAAGA     110
          :::::::: ::::::::::::::.:::: .:::::::::.:::::::::::::::::

BRT36     CCCAGCAACAATACAAGCAAAGGTATACATATAGGACCAGGGAGAGCATTTTATA     160
BRT312    CCCAGCAACAATACAAGCAAAGGTATACATATAGGACCAGGGAGAGCATTTTATA     160
BRT315    CCCAGCAACAATACAAGAAAAGGTATACATATAGGACCGGGGAGAGCATTTTATA     160
BRT14     CCTAACAACAATACAAGAAAAGGTATACATATAGGACCATGGGGCACATTTTTTG     165
          :: :.:::::::::::::: ::::::::::::::::::::::..::.: .::::::::.-

BRT36     CAACAGAAGCAATAACAGGAGATATAAGACGAGCATATTGTAACATTAGTAGAGC     215
BRT312    CAACAGAAGCAATAACAGGAGATATAAGACGAGCATATTGTAACATTAGTAGAGC     215
BRT315    CAACAGGAGAAATAATAGGAGATATAAGACAAGCACATTGTAACATTAGTAGAGT     215
BRT14     CAACAGGAAACATAATAGGAGATATAAGACAAGCACATTGTAACATTAGTAGAGC     220
          :::::::-:.  :::: ::::::::::::::.:::: ::::::::::::::::::

BRT36     AGCATGGAATGAAACTTTAGGACAAATAGTTGAAAAATTAAGAGAACAATTTGAG     270
BRT312    AGCATGGAATGAAACTTTAGGACAAATAGTTGAAAAATTAAGAGAACAATTTGAG     270
BRT315    AGAATGGAATAACACTTTAGGACAAGTAGTTGAAAAATTAAGATTAAAATTT---     267
BRT14     AAAATGGGATAACACTTTAAAAAAGATAGTTGACAAATTAAGAGAACAATTTGGG     275

          :. :::::.::.: :::::::.: :..:::::::: :::::::::.-: :::::

BRT36     AATAGAACAATAGCCTTTAATAAGTCCTCAGGAGGGGACCTAGAAATTGTAATGC     325
BRT312    AATAGAACAATAGCCTTTAATAAGTCCTCAGGAGGGGACCTAGAAATTGTAATGC     325
BRT315    AATAAAACAATAGCCTTTAATAAGTCCTCAGGAGGGGACCTAGAAATTGTAATGC     322
BRT14     AATAAAACAATAGTCTTTAATCGATCCTCAGGAGGGGACCCAGAAATTGTAATGC     330
          :::::.:::::::::: :::::::: .-:::::::::::::::::: ::::::::::::::

BRT36     ACAGTTTTAATTGTGGAGGGGAATTTTTCTACTGTAATACAACACAACTGTTTAA     380
BRT312    A---GTTTAATTGTGGAGGGGAATTTTTCTACTGTAATACAACACAACTGTTTAA     377
BRT315    ACAGTTTTAATTGTGGAGGGGAATTTTCCTCCTGTAATACAACACAACTGTTTAA     377
BRT14     ACAGTTTTAATTGTAGAGGGGAATTTTTCTACTGTAATACATCAAGACTGTTTAA     385
          :   .:::::::::.:::::::::::::: :: :::::::::::.:: .:::::::::

BRT36     TAGCACTTGGATGAATAGTAGTAATGGGGGGATTGATACTACCGCGGATGATGGA     435
BRT312    TAGCACTTGGATGAATAGTAGTAATGGGGGGATTGATACTACCGCGGATGATGGA     432
BRT315    TAGCATTTGGAGGAATAGTAGTTATGGGGGAGATTAATACTACCGCGGATGATGGA     432
BRT14     TAGTACTTGGAGGATGAATGATACTGCAAGGTCAAATAACACTGAAGGAAATGAC     440
          ::: : ::::::.:::.:.:-:. :: ---:. ..::::  :: : .:.---:::.

BRT36     AAATTCATACTCCCATGCAGAATAAAACAAATTATAAACATGTGGCAGGAAGTAG     490
BRT312    AAATTCATACTCCCATGCAGAATAAAACAAATTATAAACATGTGGCAGGAAGTAG     487
BRT315    AAATTCATACCCCAATGCAGAATAAAACAAATTATAAACATGTGGCAGGAAGTAG     487
BRT14     ACAATCATACTCCCATGCAGAATAAAACAAATTATAAACAGGTGGCAGGAAGTAG     495
          : :.:::::::: :: :::::::::::::::::::::::::::.:::::::::::::

BRT36     GAAAGGCAATGTATGCCCCTCCCATCAAAGGACAAATTAGATATTCATCAAATAT     545
BRT312    GAAAGGCAATGTATGCCCCTCCCATCAAAGGACAAATTAGATGTTCATCAAATAT     542
BRT315    GAAAAGCAATGTATGCCCCTCCCATCGCAGGACAAATTAACTGTTCATCAAATAT     542
BRT14     GAAAAGCAATGTATGCCCCTCCCATCAGAGGACGACTTAGATGTTCATCAAATAT     550
          :::.:::::::::::::::::::::. :::::.: :::. :.:::::::::::::

BRT36     TACAGGGCTGCTATTAACAAGAGACGGTGGTAATATCACAAATGAGACCGAGATC     600
BRT312    TACAGGGCTGCTATTAACAAGAGACGGTGGTAATATCACAAATGTGACCGAGATC     597
BRT315    CACAGGGCTGCTATTAACAAGAGACGGTGGCAATAACACAAACGGAGACCGAGACC     597
BRT14     TACAGGGCTGCTATTAACAAGAGATGGTGGTACTAACACGAACGAGTCTGAAATC     605
          :::::::::::::::::::::::::: ::::: : ::.:::.:: :.:.: ::.:  :

BRT36     TTTAGACCTGGAGGAGGAGATATGA---    625
BRT312    TTTAGACCTGGAGGAGGAGATATGAGGG    625
BRT315    TTTAGACCTGGAGGAGGAGATATGAGGG    625
BRT14     TTCAGACCTGGAGGAGGAGA---------    625
          :: ::::::::::::::::::
```

APPENDIX 2

A multiple alignment of the published V3 loop amino acid sequences of clones representing HIV_1 quasispecies present in patient RT3 and RT1. Amino acid homologous to the consensus sequences is indicated with periods. All amino acid were derived from nucleotide sequences (shown in appendix 1). "N°" character to designate the number of clones obtained having the indicated amino acid sequence.

(A) Sequence analysis of the V3 loop of 15 clones from patient RT3.

|←----------V3----------->|		N°
CTRPSNNTSKGIHIGPGRAFYTTEAITGDIRRAYC		B_RT3.6	
.....			14
........R.....GE.I...QR.H.		B_RT3.15	01

(B) Sequence analysis of the V3 loop of 15 clones from patient RT1.

|←----------V3----------->|		N°
CTRPNNNTRKGIHIGPWGTFFATGNIIGDIRQAHC		B_RT1.4	
.....			15

APPENDIX 3

Prediction of antigenic residues in gp120 from B_RT3.6 according to Surface plot.

(1) Residues with high (60%) and intermediate (25%) probability of surface exposure are shown as surface exposed regions.

(2). Amino acid residues analyzed for surface probabilities in B_RT3.6.

Values: Composite(60%)
File: BRT36

Surface Region	Interior Region
	1- 36
37- 41	
	42- 88 *
89- 90	
	91- 98
99- 102	
	103- 133 *
134- 136	
	137- 141
142- 143	
	144- 203 *
204- 215	

Values: Composite(25%)
File: BRT36

Surface Site No	Residues
1	4- 4
2	13- 18
3	27- 27
4	29- 29
5	35- 42
6	51- 51
7	53- 53
8	57- 58
9	60- 62
10	73- 74
11	77- 77
12	85- 91
13	98- 103
14	113- 113
15	124- 126
16	130- 145
17	168- 168
18	172- 174
19	176- 178
20	180- 181
21	189- 197
22	199- 200
23	203- 215

File: BRT36

```
                                   5                                  10                                  15
SER LEU ALA GLU GLU GLU VAL VAL ILE ARG SER ALA ASN PHE THR
                                  20                                  25                                  30
ASP ASN ALA LYS THR ILE ILE VAL GLN LEU ASN LYS SER VAL GLU
                                  35                                  40                                  45
ILE ASN CYS THR ARG PRO SER ASN ASN THR SER LYS GLY ILE HIS
                                  50                                  55                                  60
ILE GLY PRO GLY ARG ALA PHE TYR THR THR GLU ALA ILE THR GLY
                                  65                                  70                                  75
ASP ILE ARG ARG ALA TYR CYS ASN ILE SER ARG ALA ALA TRP ASN
                                  80                                  85                                  90
GLU THR LEU GLY GLN ILE VAL GLU LYS LEU ARG GLU GLN PHE GLU
                                  95                                 100                                 105
ASN ARG THR ILE ALA PHE ASN LYS SER SER GLY GLY ASP LEU GLU
                                 110                                 115                                 120
ILE VAL MET HIS SER PHE ASN CYS GLY GLY GLU PHE PHE TYR CYS
                                 125                                 130                                 135
ASN THR THR GLN LEU PHE ASN SER THR TRP MET ASN SER SER ASN
                                 140                                 145                                 150
GLY GLY ILE ASP THR THR ALA ASP ASP GLY LYS PHE ILE LEU PRO
                                 155                                 160                                 165
CYS ARG ILE LYS GLN ILE ILE ASN MET TRP GLN GLU VAL GLY LYS
                                 170                                 175                                 180
ALA MET TYR ALA PRO PRO ILE LYS GLY GLN ILE ARG TYR SER SER
                                 185                                 190                                 195
ASN ILE THR GLY LEU LEU LEU THR ARG ASP GLY GLY ASN ILE THR
                                 200                                 205                                 210
ASN GLU THR GLU ILE PHE ARG PRO GLY GLY GLY ASP MET ARG ASP
                                 215                                 220                                 225
ASN SER ARG SER ASN PRO TYR ASP
```

APPENDIX 4

(C) 1. Seroreactivity of the V3 loop peptides from gp120 clones in the HIV-1 subtypes A through F (adapted from reference 123).

TABLE 1. ELISA REACTIVITIES OF SERUM SAMPLES FROM HIV-INFECTED INDIVIDUALS IN UGANDA, NEW YORK, AND THAILAND WITH V3 LOOP PEPTIDES DERIVED FROM CLONES IN CLADES A, B, C, AND D.[a,b]

	Peptide used in assay						
Subject	*AUG06c*	*BRT1*	*BRT3*	*CUG045*	*DUG23c*	*DUG042*	*DUG044*
				Uganda			
1	++	++	+++	+++	+	+	++
2	+++	+	+++	+++	-	-	+++
6	+++	+	+++	+++	-	-	++
9	+++	-	+++	+++	-	-	+++
21	++	+	+++	+++	+	-	+++
30	+++	-	+++	+	-	-	-
32	+++	+	+++	+	+	+	+
36	+	+	+	-	-	-	-
37	-	-	+	-	-	-	+
				New York			
1	-	-	+	+++	-	-	+
2	+	-	+++	-	-	+	-
3	-	-	+++	+	-	-	-
5	-	-	++	-	-	-	-
6	+	-	+++	-	-	-	+
7	-	-	+++	+	-	-	-
9	-	-	+	-	-	-	-
14	+	-	+++	+	-	-	-
15	++	-	+++	+++	-	-	-
16	-	-	+	+	-	+	-
				Thailand			
17	-	+	-	++	+	-	-
18	-	-	+	+++	+	-	+
OD	0.03–1.23	0.02–0.87	0.12–1.79	0.01–1.89	0.04–0.13	0.04–0.34	0.08–1.68

[a]The first letter in the name of each peptide indicates the phylogenetic subtype of the parent clone.
[b]Symbols: +++, OD ≥ 1.0; ++, OD < 1.0; +, OD ≤ 0.5; −, unreactive.

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