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IMPACT OF GENETIC VARIATION ON THE SEROREACTIVITY OF LINEAR ANTIGENIC EPITOPES IN THE DIVERGENT HIV-1 SUBTYPES .

by

Gary Anthony Pestano

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

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

IMPACT OF GENETIC VARIATION ON THE SEROREACTIVITY OF LINEAR ANTIGENIC EPITOPES IN THE DIVERGENT HIV-1 SUBTYPES .

by

Gary Anthony Pestano

Adviser: Dr. William Boto

The sequence variability reported for the neutralizing epitopes of HIV-1 has presented a major obstacle in the search for candidate vaccine(s) to prevent the escalation of the AIDS epidemic. The objective of this study was to analyze the impact of genetic variation on the major neutralizing determinants in gp120 from the divergent subtypes of HIV-1. The region of ENV analyzed encodes the principal neutralizing determinant (PND) in the V3 loop and the linear CD4 binding site. This region was characterized for seven novel Ugandan clones: UG06c, UG23c, UG042, UG044, UG045, UG0963 and UG0964, and for two New York City isolates: RT1 and RT3.12. Phylogenetic analysis showed that the clones segregated with four distinct HIV-1 subtypes. UG06c and UG0964 clustered with subtype A clones, UG23c, UG042, UG044, and UG0963 clustered with the subtype D viruses, and UG045 was determined to be a subtype C clone. RT1 and RT3.12 were most related to the subtype B clones. Surface probability analyses of the amino acid residues identified at least eight potential antigenic epitopes, designated E1[C2] to E8[V5], in the divergent ENV subtypes. ELISA was conducted to determine the in-vivo availability of the predicted surface residues. The reactivities of synthetic peptides with sera from infected blood donors in Uganda, New York and Thailand, confirmed that several antigenic sites are well-conserved in the variant envelope molecules. The most intense reactivity was observed against the majority of the V3 peptides tested. However, negligible antibody reactivities were observed against at least one subset of these peptides. Secondary structure analysis indicated that the presence of a type II β–turn in the apical tetrapeptide of the V3 loop is highly predictive of the antigenicity of the PND peptides. Homology models of the tertiary conformations in the PND indicated that the epitope comprising the GPGRAF hexapeptide is specific for the group B isolates. However, the analogous epitope in many African isolates comprises the conserved IGPG tetrapeptide. This study could complement the search for broadly reactive gp120 candidate vaccines.

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INTRODUCTION

One of the urgent goals of the current search for a prophylactic therapy to control the spread of AIDS is the development of a vaccine candidate which elicits life-long sterilizing immunity from HIV. However, research efforts have thus far revealed formidable obstacles that will clearly delay the realization of this important objective. These obstacles have arisen partly from complex aspects of the life-cycle of the virus, and partly from high-risk sexual behaviors.

HIV-1 is disseminated within society via multiple mechanisms which include the exchange of infected body fluids; intravenous (i.v.) injection of contaminated blood by drug-users; exchange of semen during vaginal or anal intercourse; and transfer of infected blood or blood products into hemophiliacs. In established infections the virus is distributed within the body via cell-to-cell contact or through interactions of the cell-free virions with the target cell (1). $CD4^+$ lymphocytes, macrophages, and glial cells are the major targets for infection (1, 2). Both syncytium-inducing (SI) and non-syncytium-inducing (NSI) lymphotrophic viral strains have been characterized (1-5). It has been proposed that the NSI phenotype which is primarily found in asymptomatic individuals, is preferentially transmitted in a new infection (6), and may present a preferred target for vaccine development and efficacy evaluation (6, 7).

Infection by HIV-1 involves several viral structural and regulatory proteins. These molecules are encoded by multiple regulatory and structural genes (Figure 1a). The envelope glycoprotein gp120 (Figure 1b) binds to the CD4 molecule expressed on the target cell (Figure 2). After entry the viral nucleocapsid is uncoated, the genomic RNA is reverse transcribed to a cDNA molecule, which is then duplicated and circularized (Figure 2). The viral DNA is then transported to the nucleus for integration into the host chromosomal DNA. Integration is facilitated by another viral enzyme, integrase. Stimulation of the infected lymphocytes to proliferate has been shown to result in the

initiation of transcription of the host and viral mRNAs (1). Two classes of HIV-1 RNA transcripts are synthesized in the course of replication: one subset is translated early, and is subsequently processed to provide the structural and regulatory proteins; the second subset remains untranslated and provides the genetic material that is packaged in the emerging virions. The factors and mechanisms which determine these important steps in the viral life-cycle are presently major targets for chemotherapeutic research (1, 8, 9). In contrast, vaccine research is primarily focused on the precursor of the surface glycoproteins, gp160, or on the mature molecules, gp120 and gp41 (Figure 1b).

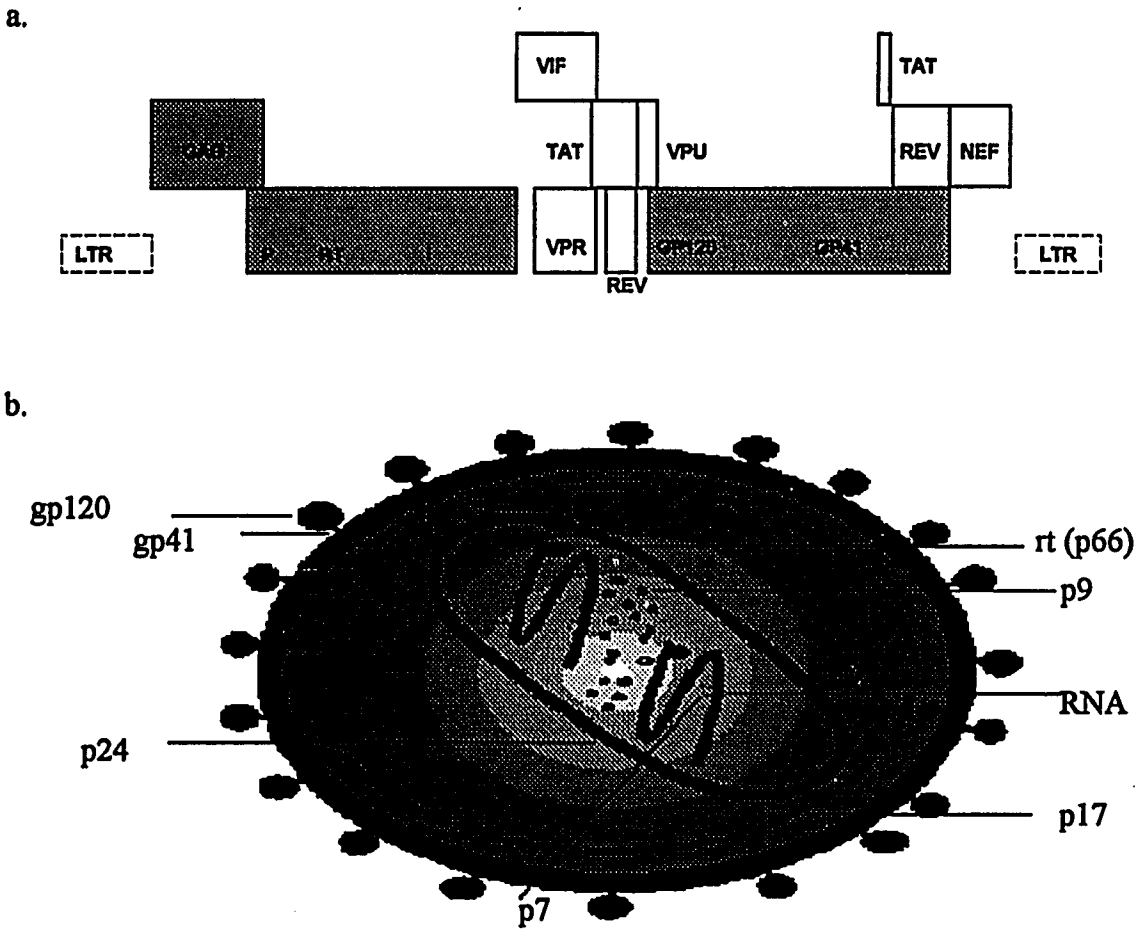


Figure 1. (a) Schematic of the HIV-1 proviral genome. (b) A cross-sectional representation of the virion. Shown are the structural proteins: gag (p7, p9, p17, p24), and gp120 and gp41, and the regulatory protein, rt (p66). The other regulatory proteins, pro, int, vpr, vif, vpu, rev, nef and tat are not shown.

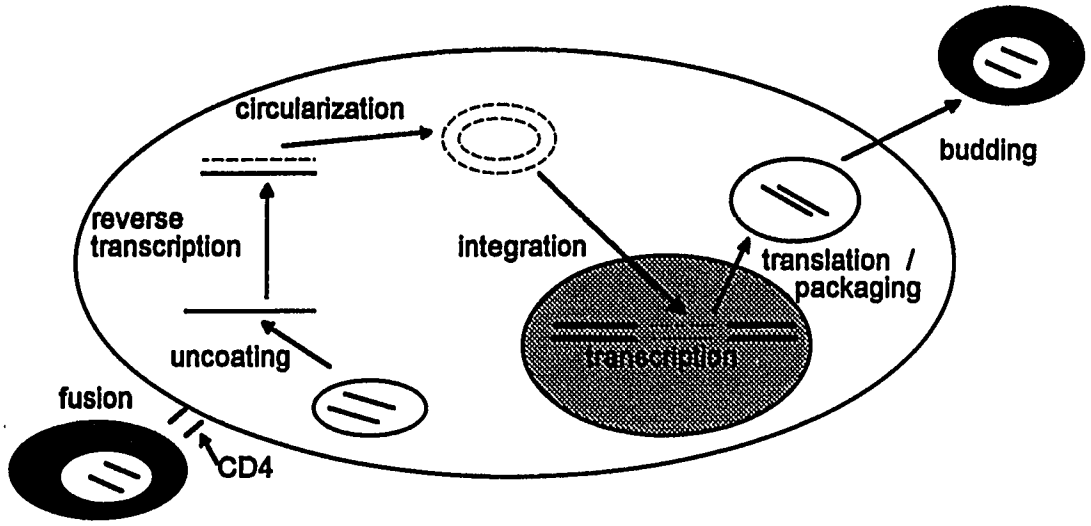


Figure 2. HIV-1 replication cycle.

Several previous findings underlie the current obstacles to the search for anti-HIV vaccines. Infection can be accomplished either by free or cell-associated virus particles (1, 9, 10); multiple cellular targets exist and tissue-specific strains have been described (11); transmission may be accomplished through multiple mucosal surfaces (1, 10); high genetic variability exists in the viral genomes (12) and consequently antigenic drift and mosaic viruses may arise (10, 12, 13); some infected cells, such as macrophages, do not express viral proteins and may serve as viral reservoirs (1, 14). Further, alternative viral receptors may exist. It was recently shown that galactosyl ceramide on neural cells (15) or on epithelial cells (16) may bind HIV-1. This could result in the expansion of the virus host range (15, 16). An additional complication arises from the apparently lengthy period of "latency" exhibited by the virus (1, 17, 18). It has now been confirmed that there is actually active replication of HIV-1 in the lymph nodes at all times in the course of the infection (17, 18).

The intrinsic genetic variability of HIV-1 and its impact on the immunogenic potential of the viral proteins is a subject of considerable interest to international vaccine research efforts (19). The viral reverse transcriptase is highly error-prone and mutations

have been predicted to occur at a rate of 0.9 to 9.26 nucleotides per genome copied (20). A significant obstacle to vaccine development is thought to arise from the mutations which accumulate in the nucleotide and amino acid sequences of gp120 in sibling clones of the virus (12, 21, 22). The number of intra-patient variants has been shown to increase as the disease progresses (Figure 3). The increase in the number of antigenic variants in an infected individual is thought to contribute to the pathogenicity of the virus (20, 23). During the progression to AIDS, the immune system may become overwhelmed by the number of circulating viral strains and antigenic variants, and it eventually collapses (20). A mathematical model has been constructed which mimics this effect when primed with data observed during disease progression (Figure 4). In this model, as in reality, the number of $CD4^+$ cells decreases dramatically as the number of antigenic variants increase, just prior to the onset of AIDS.

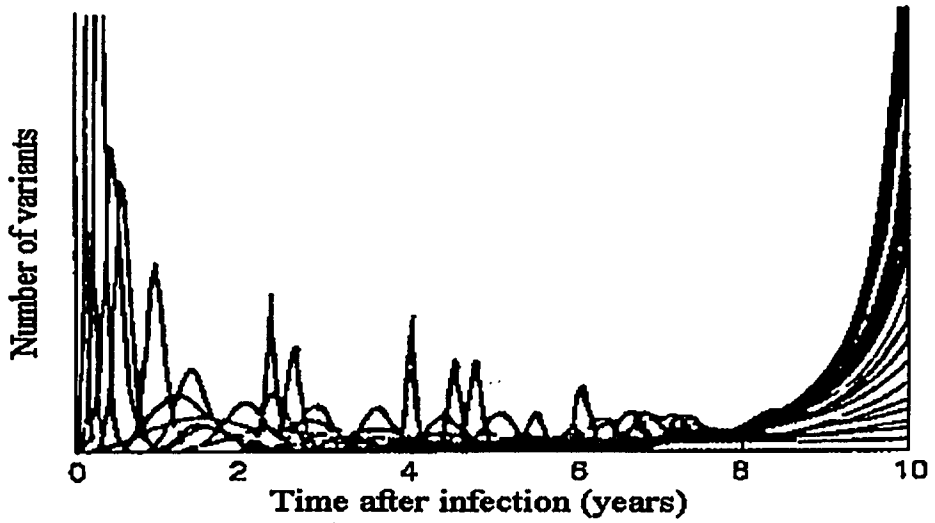


Figure 3. Antigenic diversity threshold theory of HIV pathogenesis. Each line of the graph shows the relative abundance of the HIV quasispecies in a single case of AIDS (adapted from reference 20).

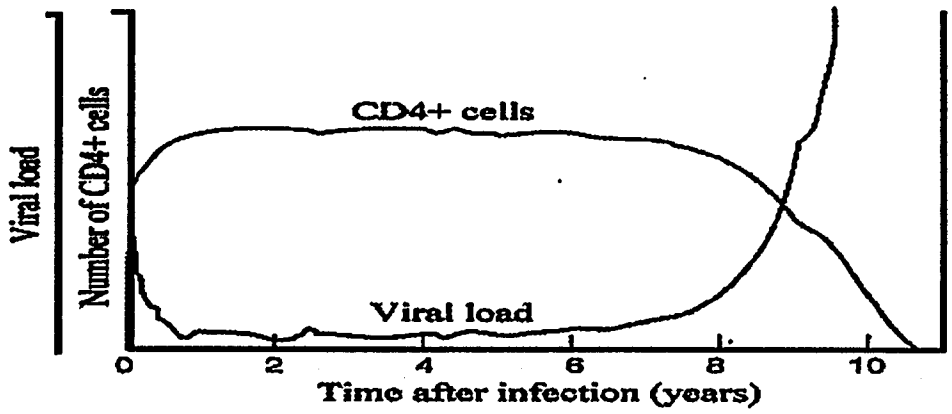


Figure 4. Computer predicted relationship of the viral load and the number of $CD4^+$ cells in an infected individual (adapted from reference 20).

Neutralization "escape mutants" have been shown to arise naturally during the replication cycle and as a consequence of selective pressure from the host immune response (23, 24). In the entire genome, the mutation rate is most pronounced for the envelope gene (ENV). Presently, gp120 (Figure 5) comprises the leading subunit candidate vaccine (12). During the natural course of infection, relatively high and sustained anti-gp120 antibody titers are observed (17, 25). However, the earliest immune response is apparently that of the cytotoxic T lymphocytes (CTLs) (25, 26). It is clear that while CTLs kill infected cells this arm of the immune system cannot clear circulating virus (25). The neutralization of cell-free virions is largely mediated by antiviral antibodies. Furthermore, complications have been observed with the CTL response generated against certain HIV-1 proteins (27). A recent report has shown that several peptides derived from HIV-1 reverse transcriptase are capable of inhibiting normal CTL responses (28). It is not yet known whether other proteins, such as gp120, also provoke a similar immunosuppressive response. In any event, it will be necessary that a vaccine(s) against HIV induce neutralizing antibodies and CTL response.

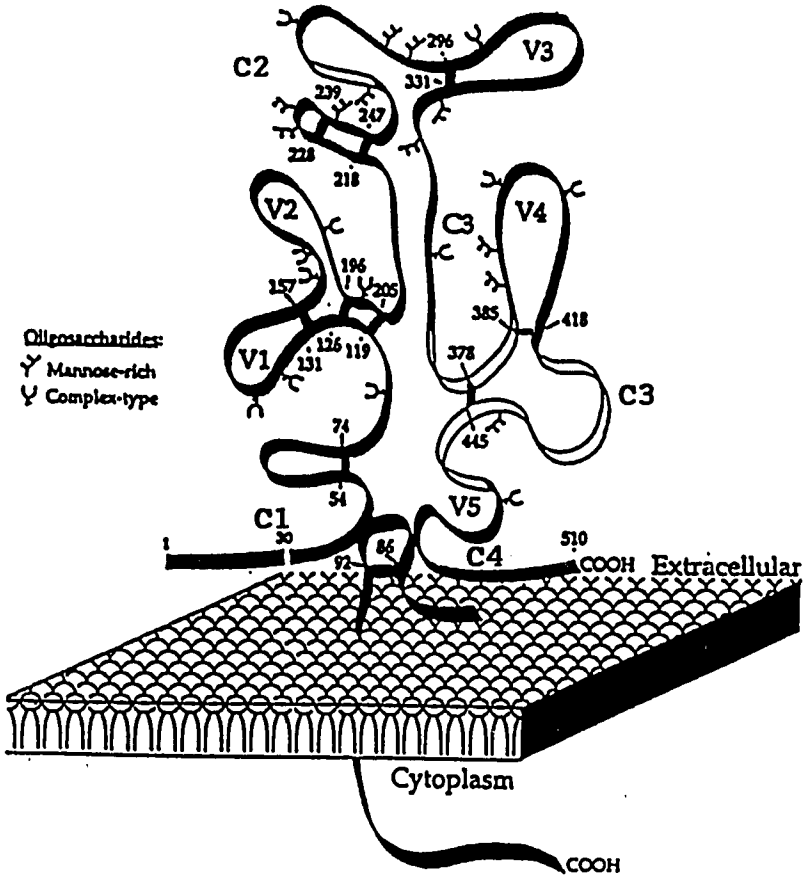


Figure 5. Model of the leading HIV-1 candidate immunogen, gp120. The variable (V) and constant (C) regions of the protein are indicated. Residues are numbered according to IIIB (12).

Phylogenetic analysis has led to the classification of ENV GP120 sequences into two major subgroups: the main cluster, "M" (subtypes A through H) and the outlier cluster, "O" (12). The isolates comprising the M subgroup account for the majority of infections thus far documented. However, the viral subtypes do not appear to be evenly distributed worldwide (7, 12, 29). This observation underscores the importance of the ongoing studies to characterize isolates from selected global sites to support vaccine research and clinical trials (Figure 6) (7, 29).

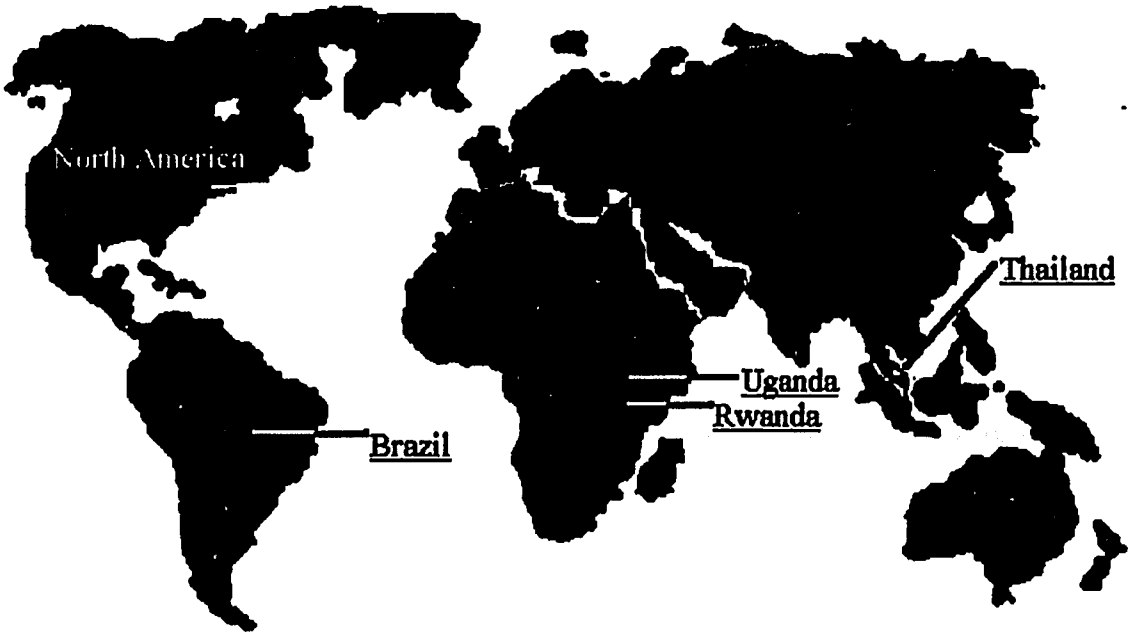


Figure 6. Map of the world showing the proposed HIV-1 vaccine trial sites (underlined) identified by the WHO.

HIV-1 strains from all of the divergent subtypes have been identified in various geographical locations on the African continent (12, 30-39). In the rest of the world, however, the prevalence of the various subtypes is more restricted: the "B" subtype is most frequently found in North and South America, the Caribbean, and western Europe (12, 40, 41); the "C" subtype has been identified in India and South Africa (28); the "E" type is found almost exclusively in Thailand (42); the "F" subtype has been described for several Romanian (43) and Brazilian isolates (44-46). The "G" and "H" subtypes have only been found in African localities thus far (36-38). Type "O" HIV-1 isolates have been identified only in individuals from Cameroon and Gabon (47, 48). Several unclassified ENV clones from diverse geographic localities have been assigned to the "U" subgroup (12). The relative distribution of the major groups will most likely continue to change with the evolution of new strains and with human migration (19, 29). In view of these findings and the chronic nature of the infection, prospects for the development of a universally

efficacious candidate vaccine(s) which elicits sterilizing immunity seem remote. Less ambitious, but more realistic goals, such as protective rather than infection-preventing vaccines, may provide some relief from the clinical symptoms (49).

The possibility of developing protective immunity to HIV-1 remains controversial. Some investigators have expressed doubt that the development of an effective vaccine for AIDS is feasible (50). Much of the disagreement stems from a lack of understanding of what constitutes protective immunity. The lack of appropriate in vivo correlates and effective animal models that can be used to quantify such a response(s) further complicates this issue (51-54). The evidence in support of protective immunity has emerged not only from planned immunization and challenge experiments conducted in animal models (55-59); but also from the analyses of the natural history of the infection (17). 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 formulations of the inactivated virions (55, 57). In several studies it was further shown that rhesus monkeys vaccinated with a nef-deficient, live SIV mutant were completely protected from i.v. challenge with a virulent strain (55, 57). Some safety concerns have been expressed that an attenuated mutant of HIV-1 similar to the nef-deletion mutant of SIV, could undergo recombination in vivo in humans, and revert to a pathogenic wild type (56, 60). Notwithstanding this reservation, the experiments with the SIV model have presented tangible evidence that some form of vaccine for AIDS may also be feasible.

The evidence from human studies in support of immunity to AIDS is mostly anecdotal. An early report had shown that i.v. infusion of hyperimmune neutralizing gamma globulin could not protect chimps from a high-dose challenge with the IIIB strain (51). Subsequent findings have indicated that such antibodies could indeed protect the animals from a low-dose challenge (61, 62). Some studies have also suggested that the presence of high titers of maternal neutralizing antibodies correlates with lower rates of

vertical transmission (53, 63, 64). Yet other investigators have presented a contrary view of the protective effects of neutralizing antibodies in prenatal infection (65, 66). Other 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 lower titers of such antibodies (53). Preliminary data reported from the use of prototype vaccines in humans has shown that rgp120 and rgp160 derived from the long-term cultured North American isolate, MN, elicits antibodies which neutralize the homologous strain (67). However these antibodies are largely ineffective against primary field isolates (68, 69). It has also been observed that serum antibodies against the autologous HIV-1 strain sometimes fail to neutralize the virus in culture (70). Many viral and host factors could account for the variations in the immune responses of the vaccinated cohorts or the infected individuals. In particular, the findings appear to support a previous observation by Schrier et. al. (71) that the in vitro proliferative response of T cells from HIV^+ cohorts is significantly affected by the HLA disparity, sequence variability and conformation of the antigenic viral peptides. Recent reports (72), including this study (34, 73), have also indicated that antibodies against the V3 loop in subtype B isolates display limited cross-reactivity with analogous peptides from isolates in other clades. It is evident that the divergence in the specificities of the major epitopes of the prevalent viral subtypes may compromise the efficacy of any candidate vaccine for global application (73). This reservation makes it prudent to anticipate reduced immunoprophylactic effectiveness of the planned vaccine clinical trials with the currently available immunogens (69, 73).

Analyses of HIV-1 nucleotide sequences (7, 12, 29-32, 34-38, 47, 48) and recent serological findings (31, 32, 34, 35) strongly suggest that the African strains of the virus are among the most divergent of the regional isolates investigated to date. In Uganda, Oram et. al. (30, 74, 75) and Albert et. al (76) have identified ENV clones that were subsequently shown to cluster with two distinct genotypes (12). Findings from the present study have shown that the prevalent isolates in Uganda actually cluster with three

divergent ENV subtypes (32, 34, 35). The immunodominant epitopes expressed in the African isolates show marked variation from the North American and European strains, as indicated by the amino acid residues comprising the major neutralizing sites in gp120 (32, 34, 35, 73). The possible impact of the variation observed in these important specificities should not be minimized in the design of the next generation of candidate vaccines.

HIV-1 is known to express at least two major neutralizing epitopes in gp120. One of these determinants is the highly variable, type-specific or group-specific PND (40, 77) located in the previously defined hypervariable V3 loop (78). The PND was defined based on the reactivities of certain neutralizing antibodies (Figure 7) (40, 77, 79-82). Neutralizing serum antibodies from naturally infected donors apparently recognize the same or closely associated specificities within the loop (83-85). Residues located at the cap of the loop, and their structural conformation are thought to be critical for the observed immunogenicity of the PND (73, 77, 83, 86). The tetrapeptide sequence GPGR, frequently occurs at this position (Figure 7). Recent NMR (87) and X-ray crystallization analyses (88, 89) have indicated that these residues assume an antigenic β-turn conformation. The DNA fragment of approximately 120 bp which encodes the V3 loop and the PND therein, is among the most variable of the sequences in the proviral genome (12). Substitutions in the PND sequences may result in the formation of type-specific neutralizing antibodies (90, 91) or antibodies with altered neutralizing specificities (92). The second major antigenic determinant in gp120 comprises the conformational CD4 binding site. This epitope(s) has been characterized by the use of a group of human monoclonal antibodies (mAb) in several recent reports (93-98). The specificity recognized by the antibodies appears to be conserved in a number of seemingly divergent HIV-1 isolates (32, 99, 100). However, the current lack of full-length gp120 sequences has hampered efforts to analyze the immunogenic potential of this epitope(s) (34). The antibodies that recognize the V3 loop and CD4 binding site may also act in synergy to neutralize HIV-1 isolates (96, 101).

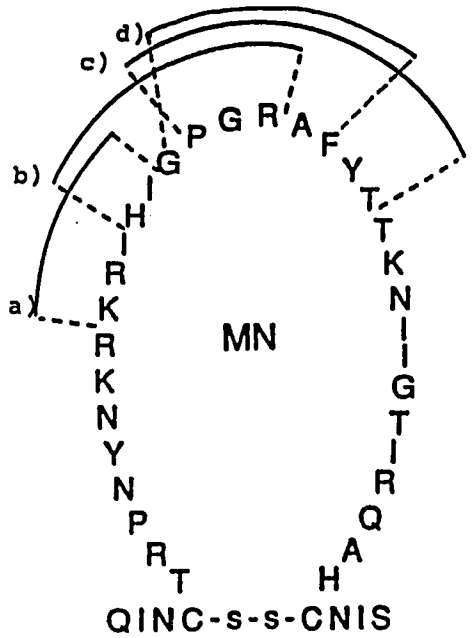


Figure 7. The V3 loop in HIV-1_{MN} showing the location of the putative PND as defined by the neutralizing activity of human monoclonal antibodies (mAbs): (a) mAb 257-2D, (b) mAb 268-11D and (c) mAb N701.9b or by (d) the mouse mAb 59.1 (adapted from reference 84).

A major aim of this study was to identify and characterize the genetic classes of HIV-1 which may account for most of the infections in the Ugandan population. These experiments involved the molecular cloning of a region extending from the C2 domain in gp120 to the N-terminus in gp41. This region was selected since it encodes several previously identified neutralizing antibody binding sites: the PND located in the V3 loop, critical residues involved in gp120/CD4 binding, and the gp120 immunodominant domain (reviewed in 102). The DNA fragment was amplified from the lysates of primary peripheral blood leukocytes (34, 41), or from short-term cultured provirus (31, 32) using the polymerase chain reaction (PCR). The PCR products were then cloned, sequenced and analyzed for phylogenetic classification. These analyses allowed for the identification of discrete viral clusters. The second aim of the study was to examine the possible impact of genetic variation on the distribution of linear antigenic sites in gp120. The amino acid residues encoded in different HIV-1 clones were analyzed for their probabilities of surface

exposure (34, 41). Complementary software programs were used in the analysis to determine the probable influence of genomic sequence variation on the surface accessibility of antigenic residues in gp120. The seroreactivity of the predicted peptides from the different HIV-1 subtypes was then assessed (34, 41). The third specific aim was to determine the influence of specific residues and peptide conformations on the seroreactivity of the divergent PND molecules (34, 73).

MATERIALS AND METHODS

(i) Sources of Test Sera and Viral Specimens

Serum samples were obtained from newly infected Ugandan blood donors reporting at clinics in Kampala: Mulago Hospital, Nakasero Blood Transfusion Service, and the Joint Clinical Research Center, and at the outpatient clinics in the Rakai district (Figure 8). Infection was confirmed using western blot and ELISA (31, 32). The New York specimens were obtained from intravenous drug users currently enrolled in a methadone maintenance program at the Addiction Research and Treatment Corporation (ARTC, Brooklyn, NY).

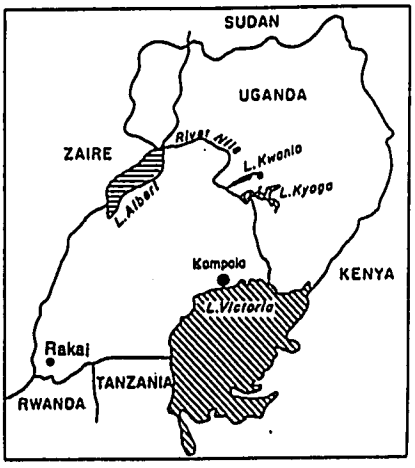


Figure 8. Map of Uganda showing the locations at which the blood samples were collected: Kampala (central) and the Rakai District (southwest).

(ii) In-vitro Propagation of Viral Isolates

Peripheral blood mononuclear cells were fractionated on Ficoll-Hypaque density gradients by centrifugation as described (103). The lymphocytes recovered from the buffy coats were cultured for 5 to 7 days at an initial density of $1x10^6$ cells/ml in RPMI-1640 (JRH Biosciences, KS) medium supplemented with 2 mM glutamine, 100 U of penicillin, 100 μg /ml of streptomycin (GibcoBRL, NY), 1% phytohemmagglutinin M (PHA-M, Difco, MI), and 20% fetal calf serum (JRH Biosciences). The primary peripheral blood mononuclear cells (PBMCs) were co-cultured with an equal number of PHA-activated PBMCs from 3 to 5 preselected normal donors for an additional 10 to 14 days, in the presence of human recombinant interleukin-2 (rIL2 [20 U/ml], Boeringher Mannheim, IN) instead of PHA-M. Normal donor PBMCs were preselected for efficient HIV-1 replication using the standard laboratory strain, $HIV\text{-}1_{IIIB}$ (31, 32). Stocks of the primary isolates were prepared after one round of infection of normal donor PBMCs. Infection titers for all stocks were determined in the microculture using 10-fold serial dilutions of the virus in 96-well plates (31). All co-cultures were fed twice weekly. Co-culture supernatants were tested for the production of HIV-1 gag gene product, p55, using antigen capture ELISA (following section viii). The stocks were stored in aliquots at -70 ^{o}C. Virus replication was determined after 14 days of culture.

(iii) Viral DNA Isolation and PCR

The proviral DNA fragment encoding the gp120 region of interest is shown (Figure 9). This segment of ENV comprises at least 625 nucleotide positions and has been shown to be suitable for the generation of reliable phylogenetic trees for the various HIV-1 subtypes (32, 34).

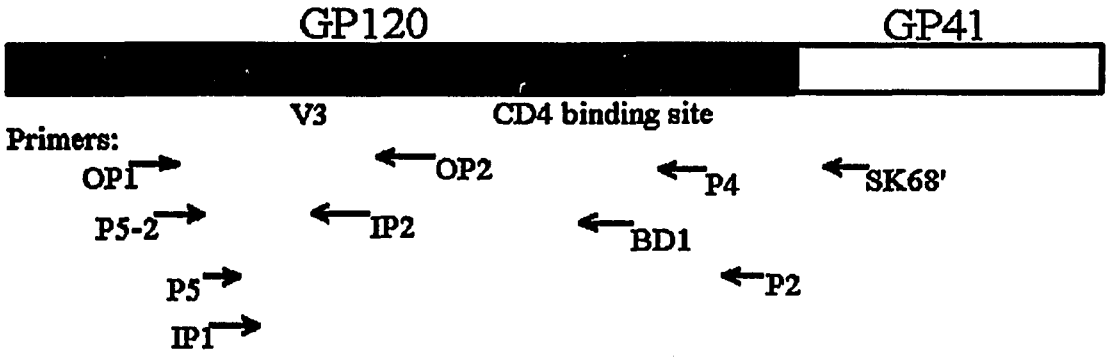


Figure 9. Linear representation of ENV showing the binding sites of the PCR and sequencing primers used in this study. The relative locations of the V3 region and linear CD4 binding site in gp120 are indicated.

Proviral DNA samples were prepared for PCR (104) using either a RT/PCR protocol for in-vitro cultured virions (32), or a DNA/RNA isolation kit (USB, Ohio) for direct amplification from donor lymphocytes (34). RNA templates were recovered from in-vitro PBMC co-cultures by centrifuging aliquots of 14-day culture supernatants at 10,000 RPM for 20 min (31, 32). HIV-1 virions were precipitated from the cell-free supernatants using polyethylene glycol (PEG-8000). Five ml of the supernatant were mixed with 5 ml of 20% PEG-800 in TE buffer [0.05 M Tris (pH 7.6), 0.05 M EDTA]. The samples were incubated on ice for 2 hr. The precipitates were pelleted at 6000 x g for 10 min. RNA was prepared from the precipitate using guanidinium isothiocyanate (105). The RNA was precipitated with ethanol, dried and dissolved in RNAse free distilled water. Fifty units of avian myeloblastosis virus RT (Promega) were added to 1 μg of viral RNA in a total volume of 20 μl of RT buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 5 mM $MgCl_2$, 20 units RNAsin, 200 μM each deoxynucleotide triphosphate [dATP, dCTP, dGTP, dTTP]) containing 15 pmol downstream primer (SK68', Table 1). The RT reaction was carried out at 42 ^{o}C for 1 hr. The tubes were then heated at 99 ^{o}C for 5 min to inactivate the transcriptase. The samples were adjusted for PCR by the addition of 15

pmol upstream primer (OP1 or IP1, Table 1) and 3 units of AmpliTaq DNA polymerase (Perkin Elmer/Applied Biosystems, CA) in distilled water containing PCR buffer (10 mM Tris-HCl, 50 mM KCl and 2 mM $MgCl_2$) in a final reaction volume of 100 µl.

Direct amplification of ENV was conducted by subjecting an aliquot (2.0 µl) of the DNA extracted from donor lymphocytes to PCR. In this experiment the primers (15 pmol of each upstream and downstream oligonucleotide) and the DNA sample were added to 1.5 units of AmpliTaq DNA polymerase and 200 µM of each dNTP (dGTP, dATP, dCTP, dTTP) in distilled water containing PCR buffer, in a final reaction volume of 50 µl. For the nested reactions, 2.0 µl of the first reaction was added to a new PCR mixture containing inner primers, and the temperature cycling was repeated. In all amplification procedures the samples were covered with an equal volume of light mineral oil (Sigma) prior to temperature cycling. The following protocol was used for the PCR reactions: denature (95 ^{o}C) 30 sec; anneal (50 ^{o}C) 30 sec; extend (72 ^{o}C) 1 min for 35 cycles; and a final extension at 72 ^{o}C for 10 min (32). All templates were subjected to an initial denaturation at 95 ^{o}C for 5 minutes.

Table 1.

Oligonucleotides used as ENV PCR primers or probes. Nucleotides in parentheses are degenerate primer positions.

Primer	Sequence	Location(Strain)	Reference
Upstream (sense)			
OP1	5'ATGTCAG(C/T)ACAGTACAATGTACAC	6491-6515(IIIB)	32
IP1	5'AAGAAGAGGTAGTAATTAGAT	6570-6590(IIIB)	32
BD1	5'TTGT(A/G)(A/G)GG(A/G)GAATTTTTCTA	6900-6921(IIIB)	32
P5-2	5'CCAATTCCCATACATTATTGT	6848-6868(NL43)	6
P5	5'ACACATGGAATTCGGCCAGTAG	6957-6978(NL43)	6
Downstream (antisense)			
OP2	5'TAGAAAAATTCCCCTCCACAA	6900-6921(IIIB)	32
IP2	5'AT(A/C)TGGGTCCCCTCC(A/T)GAGGA	6860-6879(IIIB)	32
SK68'	5'ACCAT(A/C)GTGCTTCC(A/T)GCTGCT	7342-7361(IIIB)	32
P2	5'GACGCTGCGCCCATAGTGCTTCCTG	7815-7789(NL43)	6
P4	5'ATTCACTTCTAGAATTGTCCCTC	7194-7217(IIIB)	6

Proviral DNA was obtained from single-stage or from nested amplifications. Products from the PCR were screened using 1x TBE (89 mM Trizma base, 89 mM boric acid and 1 mM EDTA, pH 8.0) agarose gel electrophoresis to identify the specimens containing proviral DNA sequences. Oligomer hybridization (106) was used to verify the sensitivity and specificity of the PCR procedure prior to molecular cloning (107). Ten picomoles of the oligonucleotide IP2, (Table 1) was end-labeled (108) with γ^{32}P ATP (S.A. 6000 μCi/mmol) and used as a probe in this experiment. The IP2 oligomer binds to a region just downstream of the V3 loop. The DNA marker was end-labeled using the same procedure. A probe mixture was prepared by adding the labeled probe (3 pmol) to 10 μl distilled water containing 24 mM NaCl and 4 mM EDTA. The PCR product was then added to the probe. The mixture was heated to 95 ^{o}C for 5 min to denature the double-stranded PCR products and then incubated at 37 ^{o}C for 15 min. The heteroduplex DNA bands were resolved on 5% non-denaturing polyacrylamide gels. The "wet" gel was wrapped in saran wrap and exposed to XOMAT-AR film (VWR, CA) with an intensifying screen at -70 ^{o}C. Oligomer hybridization with a serially diluted HIV-1 plasmid, pBH10, showed that DNA template amounts of at least 1 pg could be amplified and detected by this technique in a reproducible manner (107).

(iv). Cloning and Sequencing of PCR Products

The PCR products were ligated into the amp^{r} pT7Blue vector (Figure 10) using the 'A' overhangs produced by Taq DNA polymerase during the reaction. Taq inserts an adenosine residue at the 3' end of each new DNA strand independent of the template sequence (Novagen, WI). The PCR products were electrophoresed in 1% low melting point agarose gels. The gel fragments containing the DNA bands of the correct size were then excised and subjected to gelase digestion as suggested by the manufacturer (Epicenter Technologies, WI). Following overnight incubation with gelase at 45 ^{o}C, the DNA was precipitated with 5 M ammonium acetate and ethanol. The viral DNA fragments

were used in ligation reactions with the vector at a ratio of approximately 1:1 (32, 34). The reactions were allowed to proceed overnight at 16 ^{o}C. Three microliter samples of the ligation mix were used to transform Nova Blue competent E. coli cells (Novagen) in 100 µl of SOC. β-gal negative transformants were selected on X-gal indicator plates after overnight incubation at 37 ^{o}C. Recombinant (white) colonies were screened using PCR. In this reaction the M13 forward and T7 primers (Pharmacia, NJ), which bind to sequences flanking the cloning site in pT7 (Figure 10), were used to confirm the vector/insert recombination.

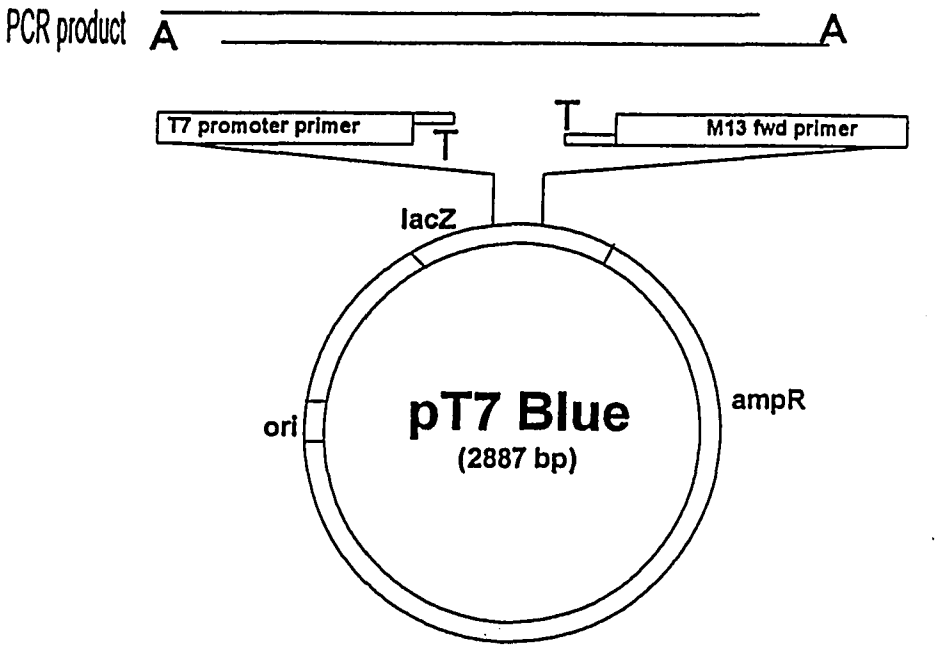


Figure 10. Map of pT7 Blue used for ligation of the PCR products. 'T' overhangs in the cloning site of the plasmid are shown.

Plasmid DNA was purified from mini-preps using the alkaline lysis method (108). The double-stranded (ds) DNA plasmids were denatured in 0.1 M NaOH, phenol/chloroform extracted, and then ethanol-precipitated. The DNA was sequenced in

both directions by the dideoxynucleotide chain termination method of Sanger et. al. (109) using α^{35}S dATP and the Sequenase kit (version 2.0, USB). Initially, the IP1 and IP2 primers (Table 1) were used to sequence the V3 loop. Except for instances where V3 nucleotide sequence variation was evident in sibling clones, only one clone was fully analyzed from each sample preparation.

Sequencing was extended throughout the cDNA fragment using the "walking primer" method (31-35). The sequencing reactions were analyzed in 6% denaturing polyacrylamide gels. The wet gels were transferred to 3 mm filter paper (Whatman, OR), vacuum dried and then autoradiographed. In subsequent experiments (34, 35, 41) an automated DNA sequencing protocol (ABI Sequencer model 373A) was implemented using Taq DNA polymerase and fluorescent dye-deoxynucleotide terminators (Prism Kit, ABI). In these reactions the ds DNA templates (1 μg per reaction) were added to a reaction mix containing the nucleotide dye-terminators, polymerase, sequencing buffer, and 30 pmol of the pertinent primer. The mixture was subjected to cycle sequencing using the following temperature cycle: denaturation at 95 ^{o}C for 30 secs and annealing/extension at 50 ^{o}C for 4 min. The cycles were repeated 25 times. The sequencing reaction products were purified from unincorporated primers and dye-terminators, using CentriSep 100 spin columns (Princeton Separations, NJ). The reactions were analyzed on 6% denaturing polyacrylamide gels as described above. Base calling and preliminary data processing were conducted using Seqed (version 1.0, Applied Biosystems).

The nucleotide sequences were analyzed using algorithms in PC\Gene (version 6.26, Intelligenetics, CA) and GCG (version 8.1, Genetics Computer Group, WI) program packages. PILEUP and PRETTYPLOT (GCG) and CLUSTAL (PC/Gene) were used to generate multiple alignments of the nucleotide residues and the deduced amino acid sequences.

(v). Phylogenetic Tree Analysis of ENV Sequences

Genetic analysis was conducted over a 625 nucleotide region of ENV (32, 34). Columns with gaps inserted to facilitate multiple alignment were removed prior to the phylogenetic analysis. The new Ugandan and New York viral DNA sequences were analyzed in relation to each other and to the reference sequences in the five major HIV-1 phylogenetic clusters (A, B, C, D and E) which have recently been reported (12). The maximum parsimony algorithms in the PAUP and DNABOOT programs in the PHYLIP package (110), were used initially to generate phylogenetic trees of envelope sequences (32). DNABOOT uses a bootstrap method to estimate confidence limits on phylogenies generated using maximum parsimony. For subsequent analysis (34, 35, 41) the MEGA program package (111) was used to generate bootstrapped neighbor-joining and UPGMA phylogenetic trees. All phylogenetic trees were rooted by sequences from SIVgab, since the true ancestor of HIV-1 is still unknown.

(vi). Prediction of Antigenic Residues in gp120

The distribution of linear antigenic sites in gp120 was determined using the algorithms in SURFACE PLOT (SP, version 1.4), and POLAR TRIPEPTIDE (PT) in PPSP (Synthetic Peptide Inc, Canada), BETATURN in PC/Gene, and PEPTIDE STRUCTURE (PS) in GCG. SP predicts high probability surface and interior sites for tripeptide residues in proteins using a composite value for hydrophilicity, flexibility and accessibility (112). PT determines high probability surface residues utilizing values for surface exposure of amino acids derived from Brookhaven National Laboratory database of protein X-Ray crystallographic structures. The PT algorithms provide a graphical semi-quantitative measure of antigenicity in peptides. PS uses the Jameson-Wolf algorithm to derive a combined value for antigenicity based on hydrophilicity, flexibility, surface probability, and secondary structure parameters (β-turns, α-helicies, β-sheets, and coils) (113, 114). Regions predicted to contain three or more contiguous "hidden" residues were

not considered to be antigenic in these analyses (34). These algorithms show a high degree of correlation in their ability to detect antigenic residues in gp120 derived from the divergent HIV-1 subtypes (34, 35, 41, 115, 116).

(vii). Analyses of Secondary and Tertiary Structures in the V3 Loop

Structural conformations appear to play an important role in the recognition of antigens by antibodies. The analysis of the primary amino acid residues was extended to an investigation of the secondary and tertiary conformations of the PND in the V3 loop encoded in the divergent viral genomes. The occurrence of the β-turn conformations indicating antigenicity was determined using BETATURN. This program has been previously used to predict antigenic residues in the HIV-1 envelope proteins (78, 114) and in other proteins (117). BETATURN uses the Chou-Fasman algorithm (117) to estimate the probability of β-turn occurrence in tetrapeptides.

Homology modeling was performed using the QUANTA (release. 4.1.1)/CHARMm 22 program package (Molecular Simulations Inc., MA) to determine possible tertiary conformations assumed by the putative PND residues. The crystal structure coordinates of the mouse mAb IgG 59.1 (89), bound to the V3 peptide from HIV-1_{MN} were imported from the Brookhaven database into QUANTA for molecular analyses. The V3 loop sequences were mutated and subjected to the energy minimization calculations in CHARMm (73). CHARMm uses empirical energy functions to describe the forces on atoms in molecules. These functions, plus the parameters for the functions, constitute the CHARMm force field. The CHARMm energy functions calculate conformational energies, local minima, barriers to rotation, energy surfaces, and time-dependent dynamic behavior. These energy functions (C_E) include internal coordinate terms and pairwise nonbonded interaction terms and can be expressed by the following equation:

$$C_E = E_{bond} + E_{angle} + E_{dihe} + E_{impr} + E_{elec} + E_{vdW} + E_{hb} + E_{cons} + E_{user}.$$

The internal energy terms include: bond length (E_{bond}), bond angle (E_{angle}), dihedral angle (E_{dihe}), and improper for planar atoms (E_{impr}). The external terms (i.e. nonbonded energy terms include: electrostatic potential (E_{elec}), Van der Waals interactions (E_{vdW}), and hydrogen bonding interactions (E_{hb}). The extra energy terms include: constraint energy (E_{cons}) and user defined energy (E_{user}). The CHARMm energy was determined for each V3 peptide bound to the antibody, and then the complex was subjected to 500 steps of minimization using the method of "steepest descents" (73). This minimization method rapidly improves a poor conformation that may include many bad contacts. The predicted structures were exported to WPDB (San Diego Supercomputing Center, CA) and Molw (version 2.5, Scripps Research Institute, CA) for further analyses. Images were rendered with RASMOL version 2.5 (118) or as kinemages (119, 120).

Homology modeling is considered to be the most reliable method of predicting tertiary structures available to date (121). Since the secondary and tertiary structures of proteins are better conserved than the primary amino acid sequence (122), when crystal structure data are used to model the tertiary folding of a homologous protein, the prediction success is significantly improved.

(viii). Serological Analyses of the Antigenic Determinants in gp120

Peptides comprising the predicted highly antigenic residues in gp120 encoded in the major subgroups of HIV-1 were tested in ELISA. The implementation of this assay allowed for the validation of antibody recognition of the predicted epitopes.

(a) Peptide synthesis.

The peptides were synthesized using the solid-phase procedure (123) on an automated peptide synthesizer (Synergy, Model 432A; ABI) with fmoc (9-fluoromethoxycarbonyl) protected amino acids, and hydroxymethylphenoacetic resin (34).

Post-synthesis cleavage of the peptide from the resin and protecting groups was conducted using an aqueous solution containing thioanisole, trifluoroacetic acid, and dithioethane (EDT) as scavengers (34). The peptide mixture was then filtered through glass-wool, precipitated in cold methyl-tertbutyl ether (MTBE, Sigma), and extracted in aqueous acetic acid. Three more washes of the solubilized peptide with MTBE was conducted to remove any remaining contaminants. Further purification was not found to be necessary for ELISA (34, 35, 41, following section ix).

(b) Expression of rgp120.

The plasmid CMVint-BL (Figure 11) was used in this study to express rgp120 from the divergent subtypes of HIV-1 for serological studies. The vector was initially constructed by Chapman et al. (124) and subsequently modified to include the gp120 encoding sequences by Xie et. al. (125). The plasmid contains the SV40 origin of replication, human cytomegalovirus major immediate-early gene enhancer/promoter (hCMV IE1) and the intron A nucleotide sequences (Figure 11). The hCMV IE1 promoter is among the strongest viral gene regulators available for transient expression of diverse heterologous proteins (125-129). Both the intron A and the human tissue plasminogen activator (TPA) signal sequences in CMVint-BL have also been shown to contribute to increased secretion of the translated protein (124, 128, 129). The recombinant protein is produced as a fusion product with the TPA signal peptide (124).

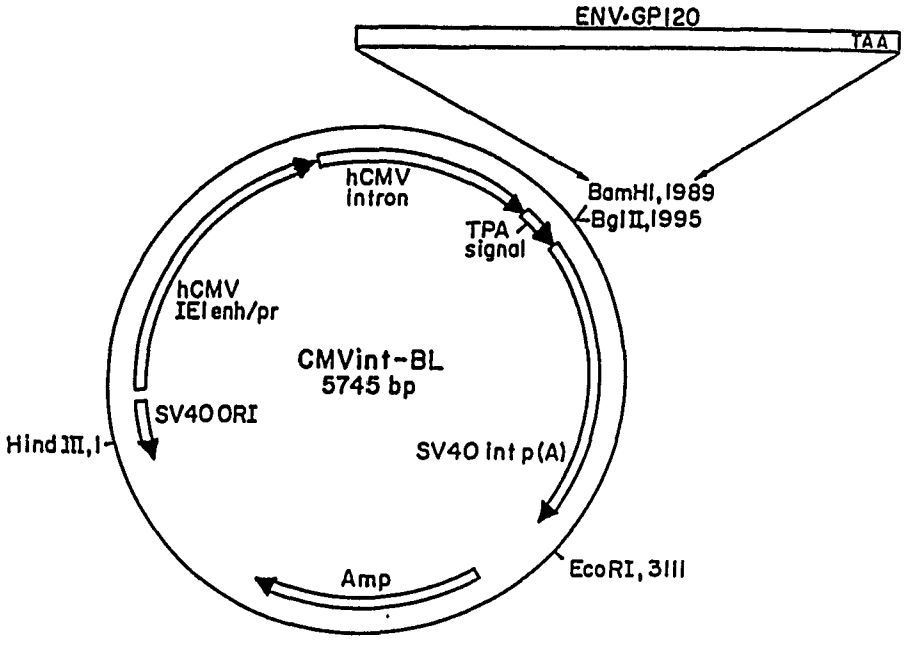


Figure 11. Map of CMV-BL used for the in-vitro expression of rgp120 from HIV-1 subtypes A, B, C and D (adapted from Xie et. al., reference 125).

CEM ($CD4^+$) cells in the log phase of growth were transfected with the respective ENV plasmids using a liposome-mediated protocol (Boeringher Mannheim). The transfection medium was prepared by adding 50 µl of HEPES (20 mM, pH 7.4) containing 5 µg of plasmid to 100 µl of DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate) (30 µg) in HEPES. The DNA was incubated in DOTAP for 15 min at room temperature (125). The entire solution (150 µl) was added to 5 ml of RPMI 1640 culture medium supplemented with 10 % fetal bovine serum, 2% penicillin/streptomycin, 0.1 mM non-essential amino acids and 2 mM L-glutamine.

Five milliliters of freshly passaged CEM cells ($3x10^5$/ml) were pelleted by centrifugation for 10 min at 250 x g. The supernatant was removed prior to the addition of the transfection mixture to the cell pellet. The cultures were incubated for a maximum of 72 hours at 37 ^{o}C in 5% CO_2 and 95% humidity. The cell cultures were harvested at 24 hour intervals, lysed, and the protein extracts were quantified by O.D. measurement at 600

nm using the modifications of the Bradford method (130) as described in the Quantify Protein Assay System (Promega, WI). The expression of the rgp120 peptides was monitored using ELISA.

(ix) ELISA

(a) Human serum ELISA.

ELISA was conducted essentially as described previously (131). Ninety six-well polyvinyl chloride microtiter plates (Baxter Scientific, NJ) were sensitized with the 125 µl of the peptides at a concentration of 50 µg/ml in 0.1 M $NaHCO_3$ buffer, (pH 9.6) for 24 hours at 4 ^{o}C. The optimal concentrations was determined by pretesting serial dilutions of the peptide with pooled human sera. This concentration of the peptides gave the best discrimination between the reactivities of the test and control sera. Duplicate or triplicate assays were conducted for each peptide.

After seven washes with ELISA buffer [15 mM Tris, 75 mM NaCl (pH 6.2) 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) 0.4% BSA] for 1 hour at 37 ^{o}C. The buffer was discarded and replaced directly with 125 µl of the test serum diluted 1/50 in fresh blocking buffer. The wells were incubated for 1 hour at 37 ^{o}C. The plates were then washed seven more times with ELISA buffer. Peroxidase-labeled polyvalent goat anti-human IgG (whole molecule, Sigma) was diluted 1/2000 in conjugate buffer (ELISA buffer containing 1/250 dilution of NGS and 0.08% BSA) (131). The plates were incubated with 125 µl of the conjugate solution for 1 hour at 37 ^{o}C and then washed seven times. Peroxidase substrate was prepared by dissolving 6 mg of o-phenylediamine dihydrochloride and 1 tablet of urea H_2O_2 (Sigma) in 15 ml of substrate buffer (10 mM Tris, 50 mM NaCl, and 1 mM EDTA [pH 3.2]). The substrate (125 µl) was reacted with the antigen/antibody/conjugate for a maximum of 30 minutes. Fifty microliters of 4 M H_2SO_4 was added to stop the reaction. Peroxidase activity was determined by O.D.

measurement at 490 nm in a microplate spectrophotometer. The respective peptides were reacted simultaneously with normal human sera to provide background absorbances. The absorbance values of the negative control values + 3 S.D. were subtracted from the experimental values to facilitate data analysis and sample-to-sample comparisons. Tests for non-specific serum reactivity were also conducted by incubating the individual serum samples with the irrelevant antigen, BSA.

(b) Mouse antibody ELISA.

ELISA reactivity of the mouse monoclonal antibody, mAb 59.1 was conducted essentially as described above with the following modifications as suggested by Xie et. al. (125). The antibody (0.6 μg) was reacted with 125 μl (50 μg/ml) of the respective peptide in 96-well microtiter plates at 4 ^{o}C (73). The binding of mAb 59.1 to the respective test peptides was assayed using peroxidase-labelled goat anti-mouse IgG (Promega) as the secondary antibody. Normal serum from BALB/c mice was used as the negative control. Data analysis was conducted as described above for the human serum samples.

(c) ELISA inhibition assay.

ELISA inhibition assay was conducted to determine the conservation of the antigenic specificities encoded in the PND from the divergent HIV-1 subtypes. In this assay, the respective competing peptides were incubated with broadly reactive human serum samples (or mAb 59.1) for 1 hour prior to the addition of the test serum to antigen-coated wells (41, 73). The percent inhibition of antibody binding to the plated antigen in the presence (or absence) of a competing peptide was computed as follows:
% inhibition = 100-[(A_{490} with competing peptide /A_{490} without competing peptide) x 100]. Competitive inhibition was used as an indicator of antigenic homology between peptides from the divergent subtypes of HIV-1 (73), and to determine the relative contribution of the putative PND sequences to antibody binding by gp120.

(x) Neutralization Assay

Virus neutralization assay was performed essentially as previously described (31, 32). Test sera were incubated at 56 ^{o}C for 30 minutes to inactivate complement. PHA-activated normal donor PBMCs at a density of $3x10^6$ cells/ml in growth medium (RPMI-1640 supplemented with 10% IL2) were conditioned by incubation at 37 ^{o}C for 1 hour. One hundred microliters of 100 $TCID_{50}$ (50% tissue culture infectious dose) of the virus was added to each well of a 96-well microculture plate. Twenty-fold serial dilutions of the test serum was added to the plated virus in quadruplicate. The plates were incubated at 37 ^{o}C for 1 hour, and then 50 µl of the conditioned target cells were added. The cell culture medium was replaced twice weekly. Production of the gag protein p55, was monitored using ELISA on day 14. Negative control wells did not contain the virus. Control virus titrations were also conducted for each experiment on the day of the neutralization assay.

RESULTS

This report describes the results of experiments undertaken to elaborate the impact of genetic variation on the predominant neutralizing determinants in gp120 encoded in the major subtypes of HIV-1. Data are presented for the new Ugandan clones, UG06c, UG23c, UG042, UG044, UG045, UG0963 and UG0964, and for the New York clones, RT1 and RT3.12 (31, 32, 34, 35, 41). All of the new clones described herein were identified during the course of these experiments. The sibling clones from RT3 (3.10, 3.11, 3.12 and 3.15) have been previously described by Riley et al. (41). The GP120 sequences in the reference isolates MN, $IIIB_{BH10}$, CM244 and RMA were similarly analyzed and the findings were compared with that for the new clones.

The selection of UG06c and UG23c for more detailed studies was based on several factors. These two isolates were previously observed to react with at least 40% of western blot positive Ugandan sera in neutralization assay (31, 32). The frequencies of sero-

reactivity suggested that these isolates or closely related strains may play a significant role in the high prevalence of infections recently reported for Uganda (132-134).

(i) PCR Amplification of ENV

DNA fragments were amplified from viral cultures using RT/PCR (31, 32) or directly from Ficoll-Hypaque fractionated lymphocytes (34, 35, 41). The use of various PCR primers for the amplification of ENV yielded DNA fragments of varying sizes: UG06c (OP1/SK68' ≈ 850 bp), UG23c (IP1/SK68' ≈ 750 bp), UG042, UG044, UG045, UG0963, UG0964, RT1 and RT3 (P5/P4 ≈ 725 base pairs) (Figure 12).

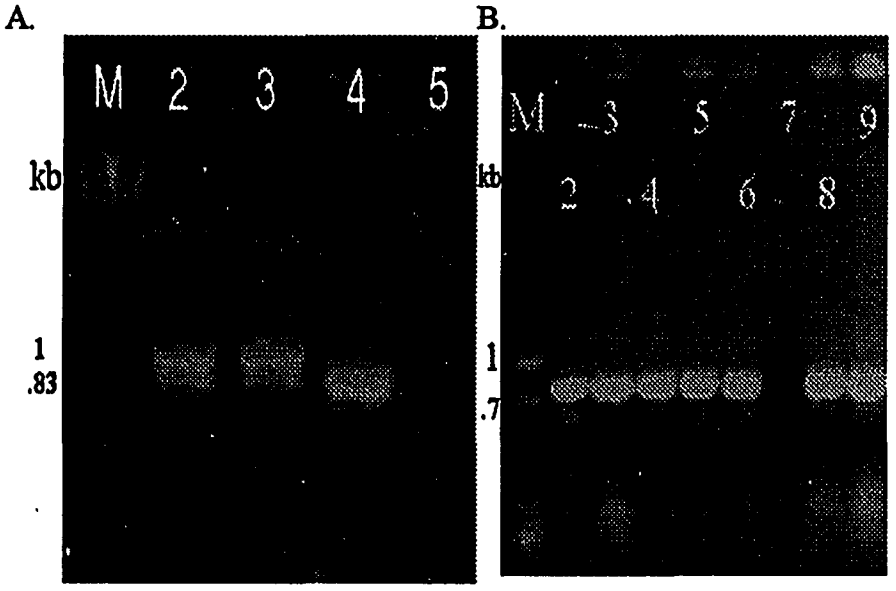


Figure 12. Agarose gels showing PCR amplified products from the Ugandan and New York City specimens. (A) UG06c (lane 2), pBH10 (3), UG23c (4) and negative control (reaction without DNA template) (5). (B) UG042 (2), UG044 (3), UG045 (4), UG0963 (5), UG0964 (6), negative control (7), RT1 (8) and RT3 (9). DNA marker (M).

Sequencing of the recombinant clones was carried out either manually (31, 32) or using an automated DNA sequencer (34, 35, 41). The nucleotide sequences have been deposited in the AIDS Research and Human Retroviruses Database (12) and in GenBank under the following accession numbers: UG06c: M98504; UG23c: M9805; UG042:

U11595; UG044: U11596; UG045: U11597; UG0963: U11598; UG0964: U11599. The sibling clones of RT1 and RT3 are listed under the accession numbers, U11586 through U11589, and U11590 through U11594, respectively.

(ii) Ugandan and New York ENV Sequences Segregate with Distinct HIV-1 Subtypes

Nucleotide sequence analyses have been used in previous studies to classify HIV-1 strains into discrete genetic clusters (12). Analyses of these clusters could conceivably provide limited targets that may be suitable for more efficient serological characterization and identification of candidate vaccines.

In this investigation, algorithms in the MEGA program package were used to generate an evolutionary tree of the new Ugandan and the New York clones in relation to the major phylogenetic clusters (A, B, C, D and E) of HIV-1 (Figure 13). The tree was rooted by the outgroup sequence from CIV_{gab}. The phylogenetic analysis of the 625 bp region of ENV indicated that the new Ugandan and North American clones clearly cluster with very different groups of HIV-1 (32, 34). The Ugandan clone UG0964 was shown to cluster with UG06c in group A, while UG042, UG044, and UG0963 were determined to cluster with UG23c in group D (Figure 13) (34). The HIV-1 sequences available in the Human Retrovirus and AIDS database (12) have shown a similar inter-group distribution of the Ugandan proviral sequences. In contrast, UG045 was observed to cluster with NOF and D760 in group C at a confidence level of 99% (Figure 13) (34). Recent analysis of envelope sequences has shown that isolates from southern and central African localities, and from India, also cluster with group C (12). However, this is the first report which describes the presence of the group C viral subtype in eastern Africa (34). The RT1 and RT3 proviral sequences derived from the New York donors, were most closely related to the reference isolates in group B (Figure 13) (34, 41). Over the 625 nucleotide region examined, the intra-subtype variation was approximately 12-13%. Inter-subtype variation ranged between 17% and 24% for nucleotides in the same region.

The nucleotide sequences for the reference clone RMA did not meet the size limitation (625 bp) required for reliable phylogenetic classification. However, this clone has been previously classified with subtype F sequences based on V3 loop nucleotide similarities (43).

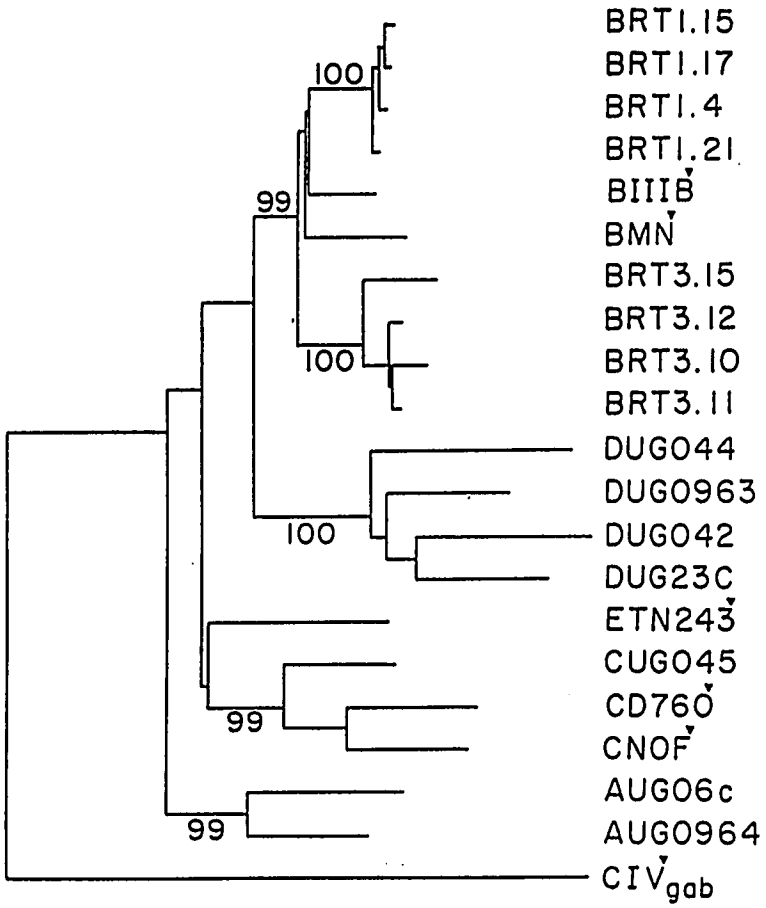


Figure 13. Phylogenetic tree showing the subtype classification of the Ugandan and New York clones developed in this study. The first letter in the name of each clone indicates its genotype[1]. Confidence levels at the nodes of the branches show the number of times out of 100 replicates the sequences to the right of the node clustered. Reference clones are indicated (▼).

[1]The names of all clones will include their subtype designations in the subsequent text.

(iii) Cross-neutralization of A_UG06c, B_IIIB and D_UG23c

An attempt was made to determine the impact of the observed sequence divergence on the neutralization sensitivity of HIV-1. The primary isolates A_UG06c and D_UG32c and the reference isolate, B_IIIB were selected for this analysis (Table 2). Serum specimens were obtained from asymptomatic subjects previously shown to be western blot positive for HIV antibodies (31). The findings from the neutralization assay showed that the Ugandan sera exhibit both type-specific and group-specific neutralizing activities (Table 2) (31, 32). One subset of the sera neutralized A_UG06c at higher titers than they reacted with either B_IIIB or D_UG23. Another serum subset neutralized both A_UG06c and B_IIIB more potently than D_UG23c (Table 2). Yet a third subset was effective against D_UG23c, but not against A_UG06c or B_IIIB. A likely interpretation of these findings is that A_UG06c and D_UG23c express conserved and divergent neutralizing epitopes (31, 32). Similar observations have been reported for neutralizing antibodies generated against other highly divergent isolates of HIV-1 (135).

Table 2.

Neutralization titers of antibodies from HIV^+ Ugandan donors against A_UG06c, B_IIIB and D_UG23c. (-) non-neutralizing serum sample.

Serum Sample	Reciprocal antibody neutralization titer		
	A_UG06c	B_IIIB	D_UG23c
6	35	-	-
22	312	-	-
1	104	-	-
3	35	35	-
9	30	104	-
11	935	35	-
4	60	104	104
7	-	-	35
14	-	-	35
19	-	-	312
2	-	-	-
12	-	-	-

(iv) Sequence Variation in Linear Antigenic Epitopes in gp120

Epitope mapping was conducted using computer algorithms to assess the potential impact of sequence variation on the distribution of antigenic epitopes in gp120 (34, 41). The SP, PT and PS analyses displayed a high degree of correlation in their predictions of antigenic residues in gp120 (34). Representative values for these predictions are shown for A_UG06c (Appendices 1A-1C). Throughout the region analyzed, eight analogous epitopes, arbitrarily designated E1 through E8, were identified in all of the clades despite the marked variation in the primary sequence (Figure 14) (34). An additional potential epitope, E9, was identified in the C-terminal residues of gp120 encoded in A_UG06c and D_UG23c and in several of the reference isolates (Figure 14). Sequences extending to the N-terminus of gp41 were not available for the other clones shown.

Over the region of gp120 examined, a high level of sequence divergence was apparent. Four of the predicted epitopes, E1[C2], E2[V3], E5[V3-V4], and E8[V5], showed conserved locations in gp120 for all of the clades analyzed (Figure 14) (34). The residues comprising E1[C2], E5[V3-V4] and E8[V5] are relatively conserved across the clades (Figure 14). The other epitopes showed noticeable levels of inter-isolate and inter-subtype sequence divergence. This variation in the primary sequence resulted in the differential amino acid composition and loss of some antigenic sites in certain clones. For instance, sequence variation presumably caused irregularity in the length and relative location of the E7[C3] site in the different clones (Figure 14) (34). The positions of E2[V3], E7[C3], and E8[V5]/E9[C4] are highly consistent with the PND, the linear CD4 binding site, and the gp120 immunodominant region, respectively (reviewed in 102). The antigenic sites E3[V3-V4], E4[V3-V4], E5[V3-V4] and E6[V4] have not been previously described (34). Accordingly, the potential of these amino acid residues to elicit neutralizing antibody is not apparent.

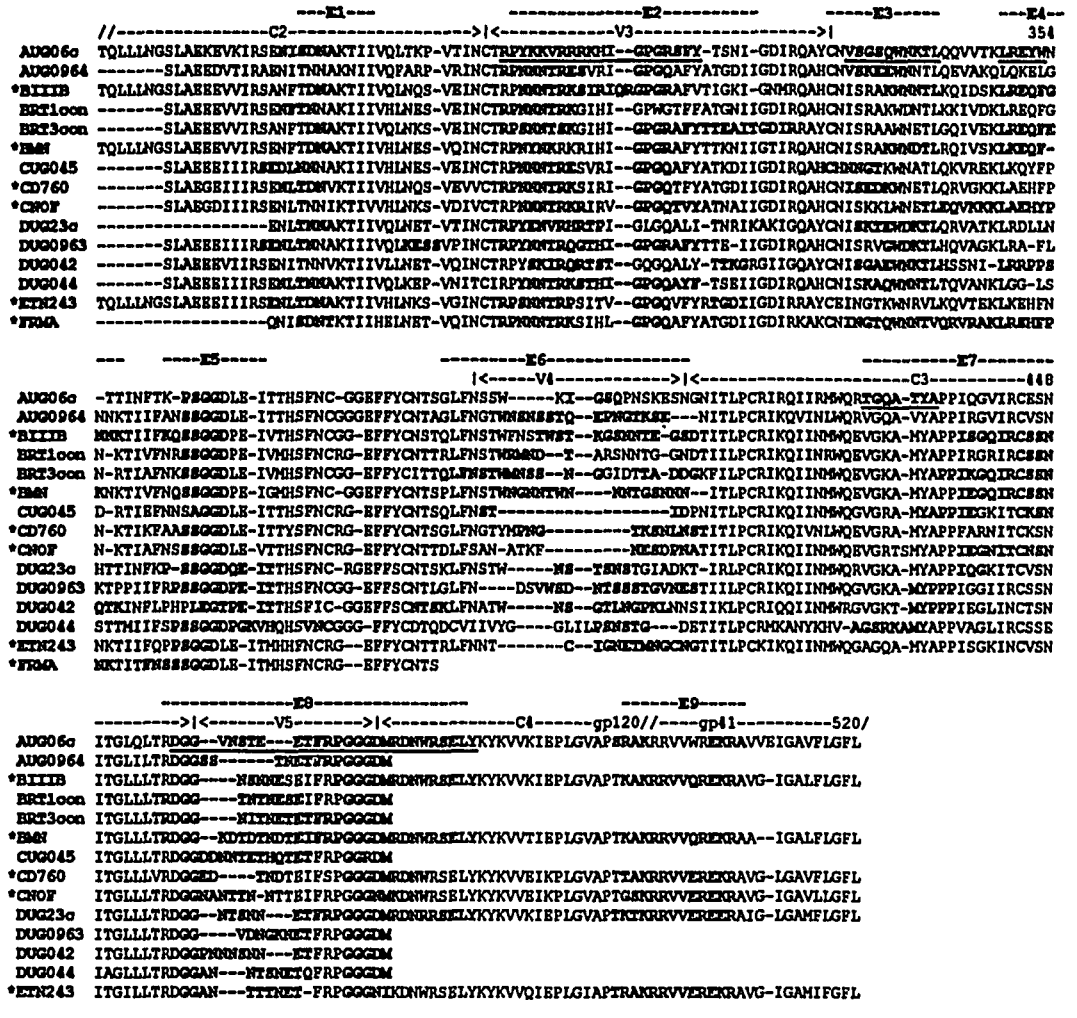


Figure 14. Distribution of linear antigenic sites in gp120 encoded in HIV-1 clones from the subtypes A through F. The predictions are according to SP, PT and PS (34). Sequences for the reference clones are indicated (*). E1 to E9 represent the predicted epitopes[2]. The domains in gp120 are designated according to Modrow et al. (78). Underlined residues were synthesized for use in ELISA; (-) gap introduced to facilitate alignment.

(v) Sero-reactivity of the Predicted Linear Antigenic Epitopes in gp120

To assess the relative surface exposure and antigenicity of the predicted epitopes in vivo, synthetic peptides comprising E2[V3], E3[V3-V4], E4[V3-V4], E7[C3], and E8[V5] in the Ugandan clone A_UG06c, were characterized in ELISA using sera from

[2] The epitope designations in the subsequent text contain the domain location in parentheses. For instance the second epitope, E2, which is located in the V3 region, is designated: E2[V3].

Ugandan, New York and Thai donors (Figure 15) (34). The peptides selected for this analysis comprised at least seven amino acid residues. The E2[V3] peptide which comprises the residues upstream of the V3 loop as well as the apical GPGR tetrapeptide, was observed to cross-react most extensively with the serum specimens (Figure 15) (34). However, the greatest degree of variation was noted in the reactivity of the individual test sera with this peptide (Figure 15). Broader reactivities, albeit of lower magnitudes, were recorded for the other peptides, E3[V3-V4], E4[V3-V4] and E7[C3]. Many of the sera tested were only weakly reactive with the E8[V5] peptide (Figure 15). The diversity in antibody reactivity observed for the peptides may be attributed to several factors: primary sequence variation, conformational restrictions in the test antigens, peptide length, or disparate host responses to a common epitope (136-138) or to the unavailability of the predicted epitopes in vivo because of tertiary and quaternary conformations in gp120 (139-141).

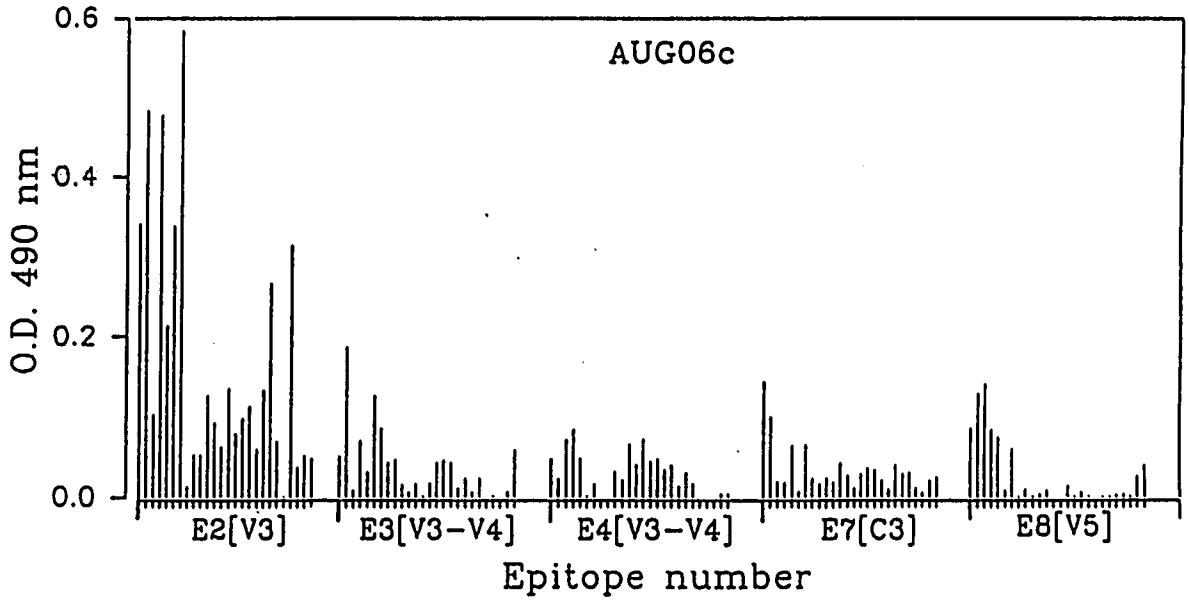


Figure 15. ELISA reactivity of individual serum samples from Ugandan (specimens 1 to 10), New York (specimens 11 to 24), and Thai (specimens 25 and 26) donors with E2[V3] (residues 297-316), E3[V3-V4] (333-342), E4[V3-V4] (349-353), E7[C3] (430-436) and E8[V5] (458-484) in A_UG06c (34). Residues are numbered according to the sequence in BH10 (12).

(vi) Sequence Variation and Secondary Structure Conformations of V3 Loop Residues

The residues comprising the putative PND in E2[V3] were further analyzed in view of the intense reactivity with sera from the diverse geographic localities (34, 73). The sequences for the V3 loop region were considerably divergent from each other and from those of the reference isolates (Figure 16). Despite extensive variation, the tetrapeptide GPGR in the cap region of the V3 loop was highly conserved in A_UG06c, B_RT3.12 and B_MN. This sequence is most frequently observed in this location in North American and European isolates (12). However, the GPGQ tetrapeptide was most represented in this series of clones (C_UG045, D_UG044, E_CM244 and F_RMA) (Figure 16). This tetrapeptide is also observed in the V3 loop of most of the HIV-1 isolates found worldwide (12). High levels of variation were apparent when the V3 loop sequences of B_RT1, D_UG23c and D_UG042, were compared to those of the other clones (Figure 16). However, the GLGQ and GQGQ tetrapeptides in D_UG23c and D_UG042 have previously been reported for a number of Ugandan (12, 31, 32, 34, 35) and other African HIV-1 V3 loop sequences (12). The highly divergent and previously unreported GPWG residues were identified in the V3 loop of the New York clone, B_RT1 (Figure 16) (34, 41).

```
Consensus       CtRP?nn?r???hiGpgqafytt??iigdIrqAhC

A_UG06c         CTRPYKKVRRRKHIGPGRSFYTSN--IGDIRQAYC
B_RT1           CTRPNNNTRKGIHIGPWGTFFATGNIIGDIRQAHC
B_RT3.12        CTRPSNNTSKGIHIGPGRAFYTTEAITGDIRRAYC
B_MN            CTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHC
C_UG045         CTRPNNNTRESVRIGPGQAFYATKDIIGDIRQAHC
D_UG044         CIRPYNNTRKSTHIGPGQAYFTS-EIIGDIRQAHC
D_UG042         CTRPYSKIRQRTSTGQGQALYTTK-GRGIIGQAYC
D_UG23c         CTRPYENVRHRTPIGLGQALITNR-IKAKIGQAYC
E_CM244         CTRPSNNTRPSITVGPGQVFYRTGDIIGDIRRAYC
F_RMA           CTRPNNNTRKSIHLGPGQAFYATGDIIGDIRKAHC
```

Figure 16. Alignment of the V3 sequences in HIV-1 subtypes A through F. The consensus sequence was determined using the majority rule with a threshold of 6: uppercase residues are identical; lowercase residues are well-conserved; (?) position is highly variant. (-) gap introduced to facilitate alignment. Bold residues are highly conserved in the V3 loop apex. The underlined residues comprising the E2[V3] site were synthesized for use in ELISA.

Variation in the primary sequences of proteins may affect secondary and tertiary conformations of antigens and thus influence interactions with antibodies (142-144) or with the T cell receptor (71). A β-turn conformation in a surface exposed region of a protein is often associated with a high probability of antibody recognition (32, 34, 117). To determine the possible influence of secondary conformations on the antigenicity of the PND, the amino acid residues comprising the V3 loop were analyzed using the BETATURN program (32, 34). Representative β-turn probability profiles for clones from subtypes A, B, C, D, E anf F are shown (Figure 17). Despite the similarities in the length of the predicted epitope in this region, the probabilities for β-turn occurrence were highly variable among the clones. The V3 loops expressing the GPGR (A_UG06c and B_RT3.12) or GPGQ (C_UG045, D_UG044, E_CM244 and F_RMA) tetrapeptides show comparably high probabilities of assuming β-turns (Figure 17). The GPGR residues have previously been shown to contribute to the PND in several North American HIV-1 isolates (40, 77, 83, 89).

The divergent residues GPWG, GQGQ, and GLGQ expressed in B_RT1, D_UG042 and D_UG23c, respectively, exhibited remarkably lower or negligible probabilities of assuming a β-turn conformation (Figure 17) (34). Given these highly divergent tetrapeptides and the apparent lack of the β-turn, it is unlikely that a highly reactive B cell epitope would be located in the V3 apex in these isolates (34, 35, 41, 116, 145).

The sequence and conformational variations observed in the PND region suggested that the clones described in this study and the reference isolates may also differ in their reactivity with serum antibodies. Such findings would be consistent with the discriminating sero-reactivities observed in neutralization assay (31, 32), and the considerable genetic distance that separates these HIV-1 isolates (32, 34). This possibility was tested by determining the ELISA reactivities of the respective synthetic peptides with a serum panel from Ugandan and North American donors.

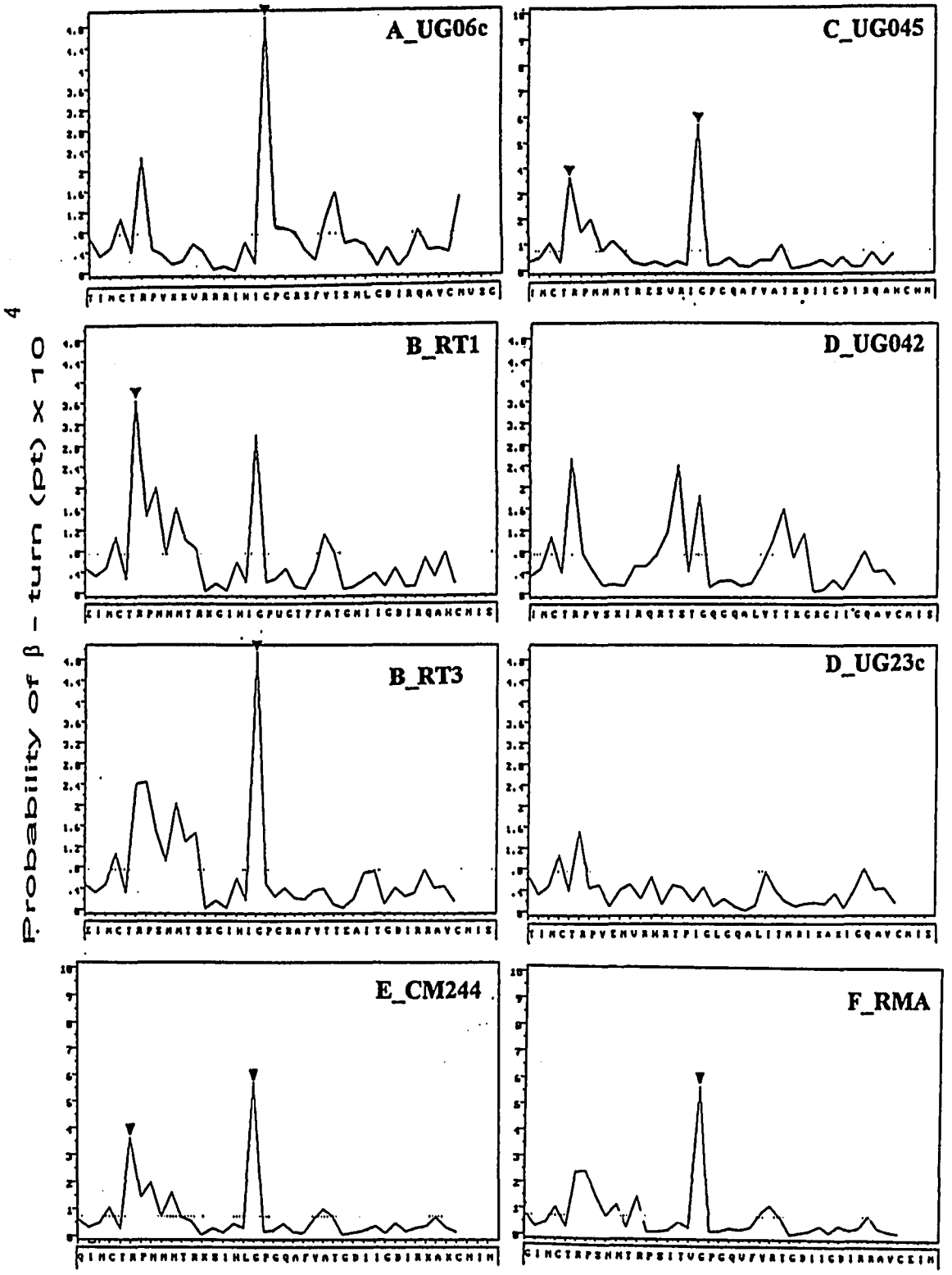


Figure 17. β-turn probability profiles for V3 loop amino acid residues in clones representing ENV subtypes A through F. Profiles were generated using the Chou-Fasman parameters (117). The locations of the most probable turns are indicated (▼).

(vii) Sero-reactivity of Divergent V3 Loop (PND) Peptides

The impact of sequence variation on the sero-reactivity of the putative PND in the divergent clones A_UG06c, B_RT1, B_RT3.12, C_UG045, D_UG23c, D_UG042, D_UG044, E_CM244 and F_RMA was examined. The reactivity of the V3 peptides from D_UG23c, D_UG042 and B_RT1 was not remarkable when tested against either the Ugandan or New York serum samples (Table 3) (34). However, the peptides from A_UG06c, C_UG045 and D_UG044 reacted intensely with a markedly higher number of the indigenous test sera (Table 3) (34, 73). Moreover, the peptides from B_RT3.12 (New York City), and from the reference clones E_CM244 (Thailand) and F_RMA (Romania), cross-reacted intensely with the majority of the Ugandan serum samples (Table 3) (73). Eleven of the fifteen Ugandan samples analyzed displayed intense cross-reactivity with at least six of the nine divergent V3 peptides tested. These findings indicated a relatively high degree of conservation of the antigenic specificities between certain V3 loop peptides. Thus no subtype-specific antigenic phenotype was observed for any of the reactive V3 loop peptides when tested with the Ugandan sera (34, 73). In striking contrast to the broad reactivity observed for the Ugandan serum samples, the North American sera showed little or no reactivity with the antigenic V3 peptides from many of the non-subtype B clones, A_UG06c, C_UG045, D_UG044, E_CM244 and F_RMA (Table 3) (34, 73).

The lack of antigenicity observed for the V3 peptides from B_RT1, D_UG23c and D_UG042, suggests that the principal neutralizing determinant is not present in this region of gp120 in these clones.

Table 3.

Seroreactivity of V3 peptides from gp120 clones in the HIV-1 subtypes A through F.

Serum samples	V3 peptide tested								
	A_UG06c	B_RT1	B_RT3.12	C_UG045	D_UG23c	D_UG042	D_UG044	E_CM244	F_RMA
Uganda									
1	++	++	+++	+++	-	-	++	+++	+++
2	++	-	+++	+++	-	+	+++	+	+++
3	+	-	+++	+++	-	-	++	+	+++
4	++	-	++	+++	-	-	+++	+	+++
5	++	+	++	+++	-	-	+++	+	+++
6	-	-	+++	+++	-	+	++	++	++
7	-	+	+++	+	-	-	++	+++	+++
10	+	+	+++	+++	-	-	+++	+++	+++
11	-	-	+	+	-	-	+	+	+++
12	+++	+	+++	+++	-	-	+++	+	+++
13	+	+	+++	+++	+	-	+++	+	++
14	+	+	++	+++	+	-	++	+++	+++
18	+	+	+++	+++	+	+	+++	+	+++
19	++	+	+++	++	-	-	+	+	+
32	+++	-	+++	+	-	-	+	+	+
New York									
1	-	-	++	++	-	-	-	++	++
2	+	-	+++	-	-	-	+	+	+
3	-	-	+++	+	-	+	+	+	+
5	-	-	++	-	-	-	++	-	-
6	+	-	+++	-	-	-	-	+	++
7	-	-	+++	-	-	-	-	+	++
8	+	-	+++	-	-	-	++	+	+
9	-	-	+++	-	-	-	-	-	+
10	-	-	-	+	-	-	+	-	+
11	+	-	+++	+	-	-	+	-	+
12	+++	-	+++	+	-	-	+++	-	+++
13	+	-	++	+	-	-	+	-	+
14	+	-	+	-	-	-	+	-	+
15	++	-	+++	+++	-	-	-	+++	+++
16	-	-	+	+	-	+	+	+	+
O.D	.03-1.23	.02-.087	.12-1.79	.01-1.89	.04-0.13	.04-0.34	.08-1.68	.02-1.69	.14-1.9

Symbols: (+++), O.D. >1.0; (++), O.D. ≤ 1.0; (+), O.D. ≤ 0.5;(-)unreactive.

(vii) Contribution of V3 Loop Residues to Antibody Binding by gp120

A transient mammalian expression system was utilized to determine the relative contribution of residues in the V3 loop to antibody binding by gp120. Recombinant gp120 from A_UG06c, B_RT3.12, C_UG045, D_UG23c and D_UG044 was expressed in the human $CD4^+$ T cell line, CEM. Maximal protein expression was detected using ELISA at 48 hours post-transfection with the respective plasmid DNA (Figure 18) (125). The crude protein extracts from the transfected cells were reacted with pooled sera from the

Ugandan donors. The selected test sera, from donors 1, 2, 4, 5, 12, 19 and 32, were previously shown to cross-react with the PND peptides from A_UG06c, B_RT3.12, C_UG045 and D_UG044 (Table 3). In contrast to the discriminatory ELISA reactivities observed in the V3 peptide assay, antibodies from the donors were capable of reacting with all of the transfected cell extracts (Figure 18) (125). Significant absorbance values were recorded against the protein extract from the cells transfected with CMV/D_UG23c. However, the most intense reactivity was observed with the protein from the CMV/B_RT3.12 transfected cells. Similar intensities of antibody reactivity were observed against the recombinant proteins from cells transfected with the A_UG06c, C_UG045 , D_UG23c and D_UG044 plasmids. ELISA inhibition assay with the homologous E2[V3] peptide, also indicated that 11% to 58% of the antibody binding to rgp120 may be directed against this region (Figure 19). This finding correlates well with a previous report which indicated that in natural infections V3 antibodies may comprise a significant proportion of the humoral response against gp120 (146).

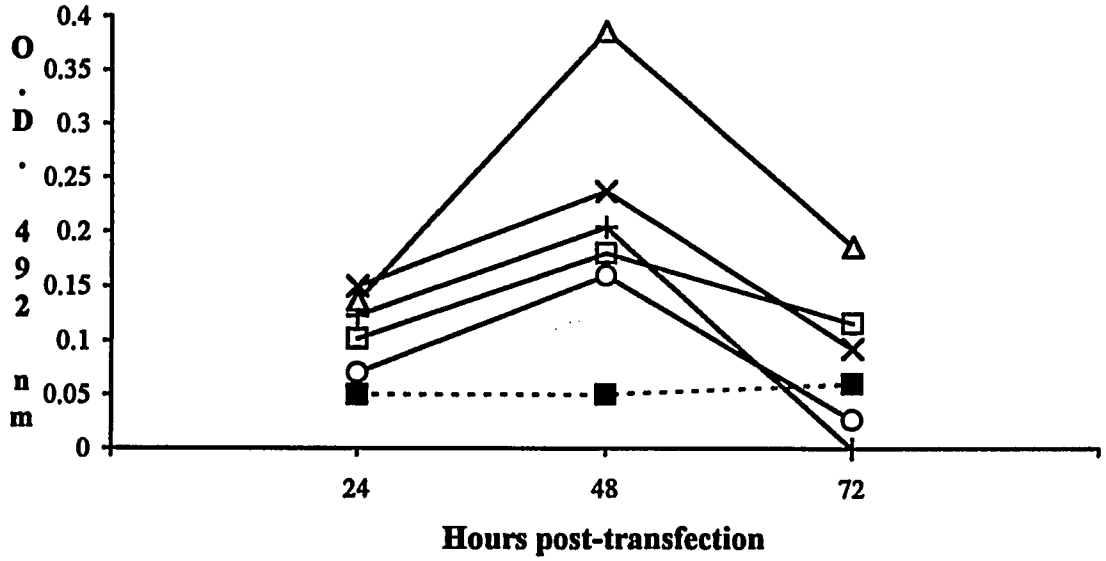


Figure 18. ELISA reactivity of the cell extracts from CEM ($CD4^+$) cells transfected with CMV/ENV: A_UG06c (□), B_RT3.12 (Δ), C_UG045 (+), D_UG23c (x), D_UG042 (O). Negative control transfection reactions were conducted with the CMV vector containing no insert (■). Sera from the $HIV\text{-}1^+$ Ugandan donors were used as the primary antibody.

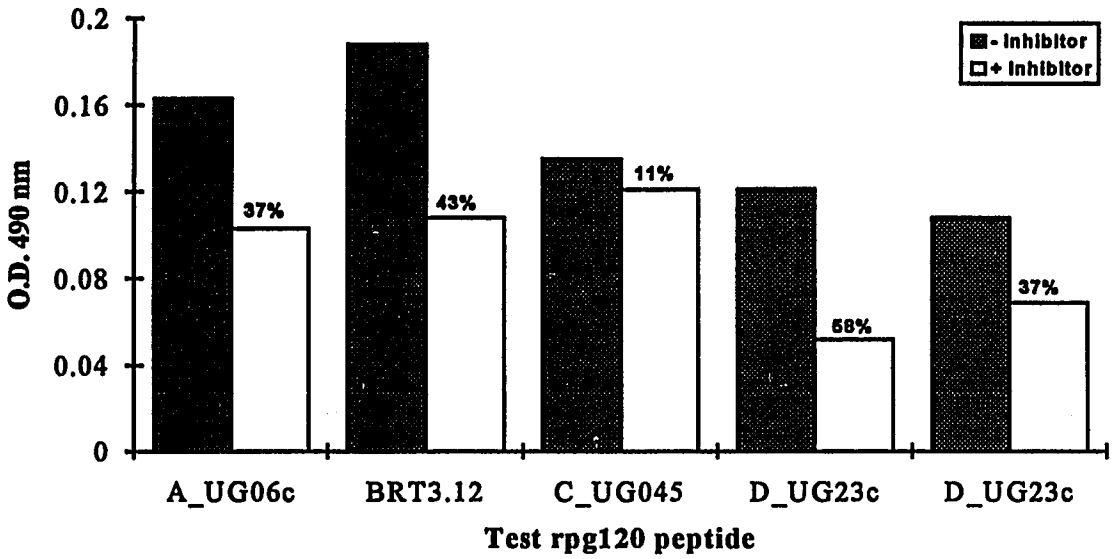


Figure 19. ELISA inhibition showing the relative contribution of the V3 residues to human antibody reactivity with gp120. Percent reduction in binding is indicated in parentheses. Open bars (□) represent the reactivity of gp120 in the presence of the homologous V3 peptides and filled bars (■) show gp120 reactivity in the absence of the inhibitor. Background values obtained from the reactivity of untransfected cells were subtracted prior to data processing.

(ix) The Majority of HIV-1 Isolates Encode Conserved Antigenic Residues in the PND

An attempt was made to identify the critical residues involved in the PND reactivity with antibody. Overlapping peptides comprising the flanking and apical region of the V3 loop in B_RT3.12, and C_UG045 were synthesized and tested for reactivity in this experiment (Figure 20). The peptides were tested with the broadly reactive serum samples from the infected Ugandan donors. For the majority of the serum samples tested, comparable reactivities were observed against the peptides comprising either P1 (E2[V3]), or the carboxy-terminal residues (P2) of the loop (Figure 20) (147, 148). These findings are consistent with reports suggesting that the antibody binding residues that comprise the PND may be conserved in divergent HIV-1 isolates (72). Indeed, competitive ELISA conducted with heterologous E2[V3] peptides clearly indicated that C_UG045 and F_RMA display analogous antigenic specificities (41) which may also be expressed in

A_UG06c and B_RT3 (Figure 21) (73). The levels of cross-inhibition by the heterologous peptides ranged from 37 % to 100 % (Figure 21). In contrast, the peptides from A_UG06c and B_RT3.12 displayed more restricted reactivity and primarily cross-reacted with each other (Figure 21) (73). These data indicate that the conserved residues, IGPG, located in the apical region of the V3 loop, may comprise the minimal requirement for antibody binding to divergent gp120 molecules (73).

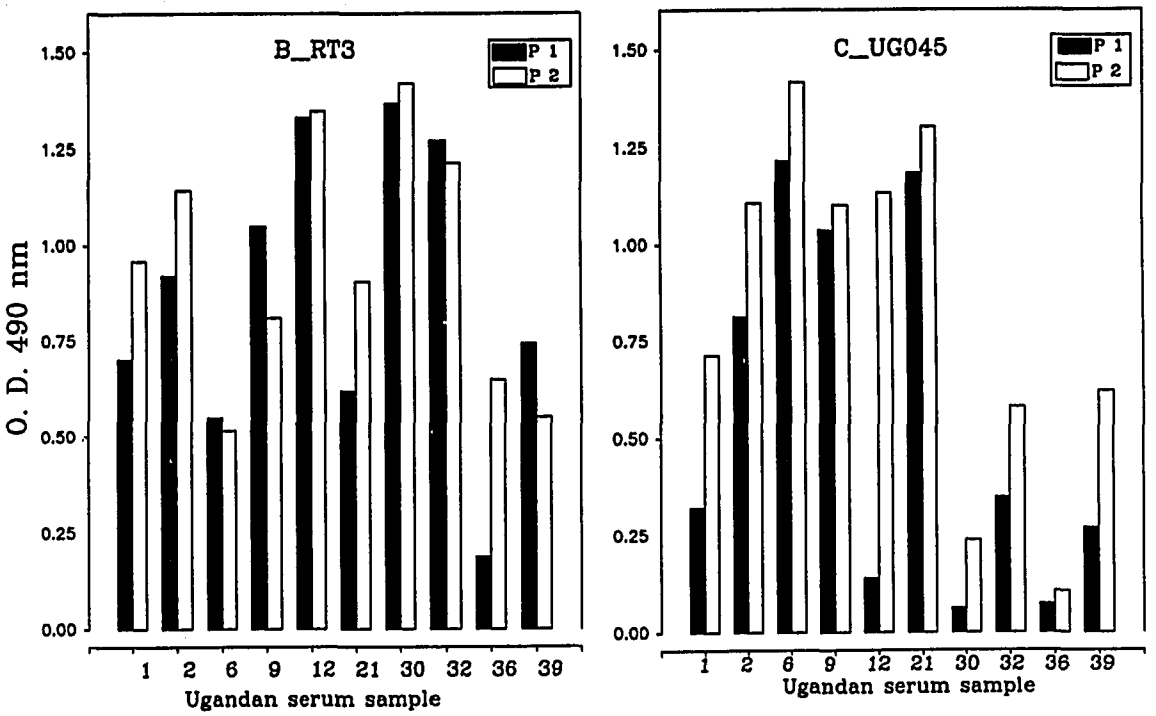


Figure 20. ELISA reactivity of overlapping V3 peptides from B_RT3.12 and C_UG045 with individual serum samples from HIV^{+} Ugandan donors. Sequences of the peptides are shown below the histograms. Residues in the region of overlap are underlined.

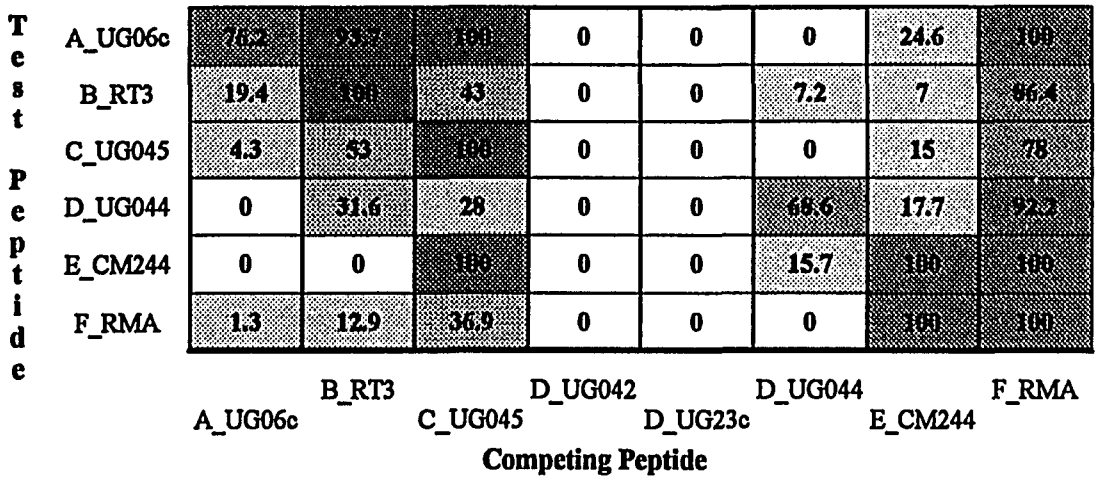


Figure 21. ELISA inhibition of human polyclonal serum reactivity with the E2[V3] peptides from subtypes A through F (73). Peptide inhibitory activity by heterologous V3 peptide is indicated as a percentage of the absorbance values in the absence of the competitor (see Methods and Materials). The more intense shading indicates a higher percentage of inhibition. A_{490} values in the absence of the competing peptide were: A_UG06c (0.362), B_RT3 (0.53), C_UG045 (0.468), D_UG044 (0.368), E_CM244 (0.215) and F_RMA (0.504).

(x) MAb 59.1 Displays Subtype-specific V3 Peptide Reactivity

ELISA reactivity of the subtype B monoclonal antibody, mAb 59.1 provided additional evidence for antigenic disparities in the major epitopes recognized by polyclonal antibodies from the African and North American donors. The epitope recognized by mAb 59.1 comprises the highly conserved GPGRAF tetrapeptide (89). The antibody is thus capable of neutralizing a number of HIV-1 isolates which may express otherwise variable flanking V3 loop sequences (89, 149). Consistent with these reports, it was observed that 59.1 reacted intensely with the V3 peptides from B_MN and B_RT3.12 (Figure 22) (73). However, the antibody did not react with the E2[V3] peptides from heterologous subtypes C_UG045, D_UG23c, D_UG042, E_CM244, or F_RMA. Further, only the B_RT3 peptide was capable of inhibiting mAb 59.1 binding

to B_MN (Figure 22). These findings indicate that the highly conserved residues GPGRAF located in the cap of the V3 loop may comprise a common epitope(s) only for antibodies developed against gp120 from the subtype B isolates.

Test Peptide	MN	BRT3	CUG045	DUG042	DUG023	E_CM244	F_RMA
B_MN	70	19	0	0	0	0	0
B_RT3	[illegible]	66	0	0	0	0	0
C_UG045	0	0	0	0	0	0	0
D_UG042	0	0	0	0	0	0	0
D_UG23c	0	0	0	0	0	0	0
E_CM244	0	0	0	0	0	0	0
F_RMA	0	0	0	0	0	0	0

Competing Peptide

Figure 22. Competitive ELISA reactivity of V3 peptides with mAb 59.1. Peptide inhibitory activity is indicated as a percentage of the absorbance values in the absence of the competitor (see Materials and Methods). The more intense shading indicates higher percentage of inhibition. A_{490} values in the absence of competing peptide were: B_MN (1.825), B_RT3 (1.696), C_UG045 (0.0), D_UG042 (0.0), E_CM244 (0.0) and F_RMA (0.0).

(xi) Carbon Backbone Conformations are Conserved in Divergent V3 Peptides

Homology modeling of the tertiary structure in divergent V3 peptides was conducted to determine the possible impact of molecular conformation on antibody recognition of the PND. The crystal structure of mAb 59.1 complexed with the PND peptide from B_MN (Figure 23) was used as the precursor for the homology models (73). The peptide in HIV-1_{MN} displays the identical sequence with that of B_RT3.12. The peptide binds in a relatively flat-shaped pocket of the fragment antibody binding (Fab) region, making contacts with all complementary determining regions (CDRs) of the antibody, except for CDR L2 (89).

The GPGRAF hexapeptide in B_MN (and in B_RT3.12) forms an S-shaped conformation (Figure 24a) consisting of a type II β-turn (GPGR) followed by a type III β-turn (GRAF). The residues, RAFY, also adopt a type I β-turn (89). The NH groups from Gly^{P319}, Gly^{P321} and Ala^{P323} each form a hydrogen bond with the Fab. The side chain of Arg^{P322} is perpendicular to the plane of the S-shaped conformation and is inserted deep into the antibody combining site (Figure 23), where it make two hydrogen bonds and a salt bridge with residues from CDR L3 (89).

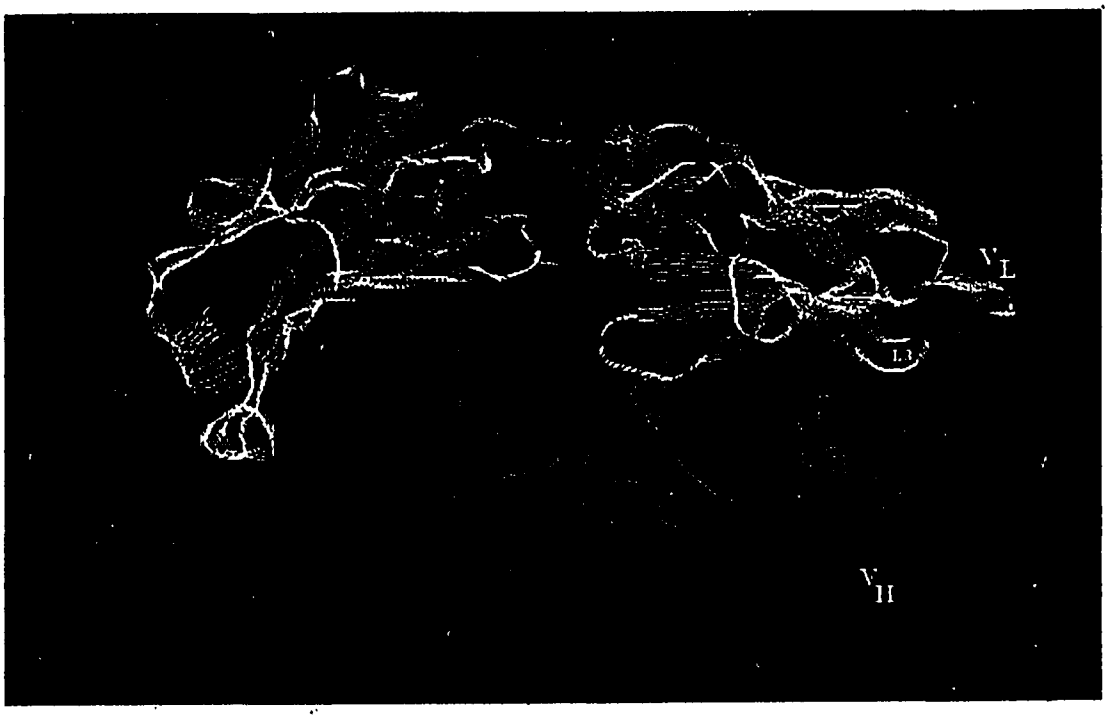


Figure 23. Fab in mAb 59.1 complexed with the PND in B_MN. The variable light (V_L) and variable heavy (V_H) chains of the antibody are represented by ribbons. The peptide is shown in red (wireframe model) with all atoms on. CDR L3 in the light chain of the antibody is indicated. The 1acy.pdb file was retrieved from the Brookhaven database and displayed in RASMOL.

Energy minimized models of divergent V3 peptides in B_MN, C_UG045, D_UG23c and D_UG042 indicated that these molecules may form analogous backbone conformations (Figure 24) (73). Mutations in the V3 peptide from B_MN to the

C_UG045 or D_UG23c or D_UG042 sequences may result in the loss of the type I (RAFY) and the III (GRAF) β-turns (Figure 24). However, the type II β-turn formed by the GPGR residues may be maintained in the GPGQ tetrapeptide (89). Gln, Arg and Lys may maintain the type II turn since the side chain NHε can form an equivalent interaction with the main chain carbonyl oxygen of Gly^{P319} at the first residue of the turn (150). Thus the R→Q substitution position i + 3 is the only substitution observed herein which may be tolerated for the type II β-turn to be formed by the core GPGx tetrapeptide.

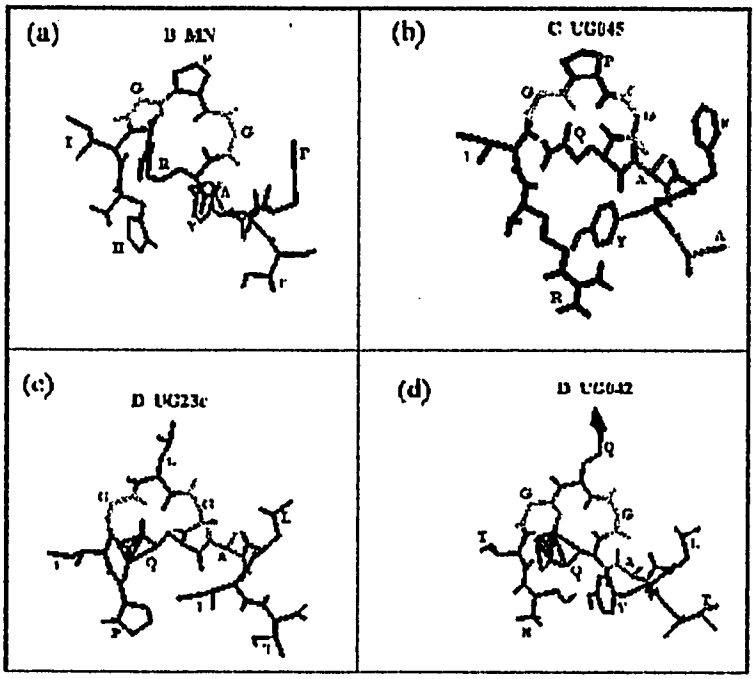


Figure 24. Predicted molecular conformations of the V3 peptides in (a) B_MN (B_RT3.12), (b) C_UG045, (c) D_UG23c and (d) D_UG042 (73). Coordinates for the "P" chain from 1acy.pdb were imported into QUANTA and mutated to generate the peptides for C_UG045, D_UG23c and D_UG042 (see Materials and Methods). Residues are colored by type.

Substitutions at i + 1, such as P→L (D_UG23c) or P→Q (D_UG042), may not be acceptable for recognition by the antibody. This is supported by the predicted conformation shown for the V3 cap residues in D_UG23c and D_UG042 (Figure 24).

Mutational analysis and antibody binding assays in ELISA have shown that the replacement of the proline at i+1 is not permissible (89). Pro^{320} is the most favored residue at this position because its inherent restriction in θ to about $^{-}60^{o}$ corresponds well with the (i+1) requirement for type II β-turns (89). The P→L and P→Q substitutions will most likely result in the loss of the type II β-turn at this position (73). The side chain from leucine and glutamine may also provide steric hindrance to antibody binding (Figure 24).

Mutations in the V3 loop apex then do not appear to significantly change the backbone conformation (α–carbons) of this region (73, 148). Further evidence of this was observed from homology models and superimposition of the predicted structures of the V3 cap residues in B_MN and C_UG045 (Figure 25) (73). The appended structures showed a high degree of conservation in the peptide backbone conformation, with variation in the antigenic structures due mostly to the side chains of the amino acids (Figure 25). However the replacement of R^{322} (at i + 3) with Q in C_UG045 may not allow critical interactions with CDR L3 in mAb 59.1 (73). The side-chain from Gln apparently does not extend as far into the antibody combining site as does the side-chain from Arg (Figure 25) (73).

Figure 25. mAb 59.1 complexed with the PND in B_MN and C_UG045. V_L and V_H in the Fab region are shown as blue and green Cα traces, respectively. The peptides in B_MN and C_UG045 are shown in red and yellow wireframe traces, respectively. The side-chains from R^{322} and Q are indicated (arrow). The structures were superimposed using the least squares method of Hendrickson in WPDB.

(xii) Lack of Correlation between V3 ELISA Reactivity and HIV-1 Neutralization

To determine whether antibody reactivity in ELISA with the V3 peptides may be indicative of neutralizing potency, neutralization assay was conducted with selected Ugandan serum samples and the indigenous isolate, A_UG06c. The sera from donors 6, 11, 12, and 32, were selected for this analysis because of their contrasting ELISA reactivity profiles (Table 3) (34). Serum samples 12 and 32 displayed intense reactivity with the E2[V3] peptide from A_UG06c, while samples 6 and 11 were only weakly reactive with the same test peptide. The data from the neutralization assay indicated that the ability of antibodies to block virus replication may not be inferred from the ELISA reactivity of the V3 peptides (Figure 26). Polyclonal antibodies from donor number 12 were observed to neutralize A_UG06c to a titer of 1/640, but serum antibodies from the other ELISA-reactive specimen, donor number 32, failed to block replication of this isolate (Figure 26). However, the serum samples which did not display ELISA reactivity (samples 6 and 11), were also incapable of neutralizing A_UG06c (Figure 26).

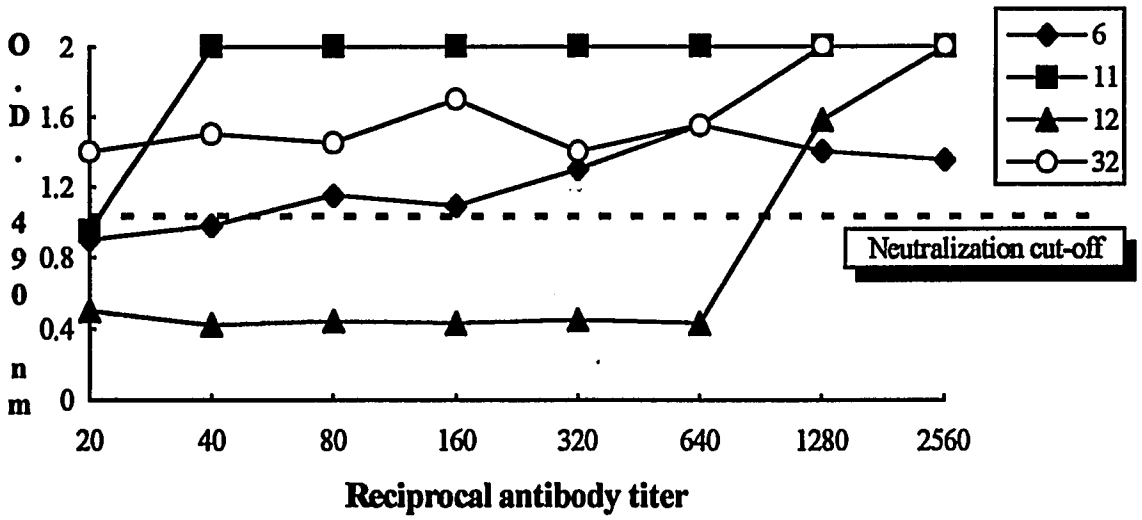


Figure 26. Neutralization of A_UG06c by antibodies from the Ugandan donors 6, 11, 12 and 32. A_{490} readings indicate gag p55 production as measured using ELISA (see Materials and Methods). The cut-off for virus neutralization was defined as 2 x (A_{490} observed for normal PBL cultures without virus) = 1.05. Virus was used at 100 $TCID_{50}$ in the assay.

DISCUSSION

The extent of serological cross-reactivity among the various HIV-1 subtypes is an important question to consider in vaccine research (19, 27). This study was undertaken to analyze the effects of sequence variation on the distribution, conformation and sero-reactivity of the linear antigenic structures encoded in the envelope protein, gp120. Highly variant gp120 sequences from primary isolates or from ENV clones of Ugandan, North American, Thai and Romanian origin were analyzed (31, 32, 34, 35, 45, 73, 125). While cross-neutralization assay offers the possibility of determining the divergences in the antigenic properties of the viral subtypes (150, 151), an attempt to classify the virus based primarily on this technique would be subject to several drawbacks. In many cases neutralization assay would be economically unfeasible in view of the large number of distinct viral subtypes thus far identified in different geographic localities (32). In addition, the scarcity of standardized serological reagents and the reproducibility of the assay in its present form are not encouraging (152). In contrast genetic analysis has been used to classify the HIV-1 strains into discrete clusters (12, 153, 154). Specifically, phylogenetic tree analysis has been conducted to classify proviral DNA clones into different groups and subgroups based on their relatedness at the nucleotide sequence level (12). A similar approach was used to identify the major subtypes of the Ugandan virus in this study (31, 32, 41). Such clusters could provide limited targets that may be suitable for more efficient immunological characterization of the HIV-1 quasispecies.

DNA fragments from the novel Ugandan and New York HIV-1 strains were PCR-amplified, cloned and sequenced (31, 32, 35, 41). The region of ENV analyzed displayed significant sequence variation and the Ugandan clones were classified with other subtype A, C or D sequences (Figure 13) (32, 34). The New York clones were also quite distinct from each other (16% divergence); however both clones clustered with the group B

viruses (34, 41). The sequences of the reference isolates from Thailand and Romania were previously determined to cluster with subtypes E (42) and F (43), respectively.

The prediction of potential antigenic residues in the divergent molecules was based on algorithms for hydrophilicity, accessibility, flexibility and secondary structures (reviewed in 122, 136, 155-159). These parameters have previously been shown to predict the occurrence of antigenic sites in gag (114), gp120 (86, 78, 114) and in other proteins (160) with at least 65% accuracy (122, 156, 160). Nine analogous B cell epitopes were defined in the region extending from the C2 to the N-terminus of gp41 (Figure 14) (34, 35, 41). A similar analysis of gp120 sequences expressed in the IIIB clones has yielded antigenic profiles (78) which correlate well with those described in this study. As expected, a relatively high number of variations was noted for the amino acids comprising many of the predicted epitopes (Figure 14) (34). E2[V3] and E6[V4] were determined to have the highest degree of variability. Immunoassays have been observed to markedly improve the reliability of this method of epitope mapping (161). A similar approach was used in this study to validate the predictions and to analyze the antigenic properties of the various gp120 peptides from the divergent clades of HIV-1. The ELISA analysis indicated that despite individual variations in the serum reactivities, all of the predicted antigenic peptides in the Ugandan isolate, A_UG06c, were capable of reacting with antibodies from donors likely to be infected with divergent isolates (Figure 15) (34). These data strongly suggest that despite extensive inter-isolate sequence heterogeneity, analogous linear regions in gp120 are generally conserved in terms of their surface probability (34, 35, 41).

Surprisingly, E2[V3] showed a greater degree of cross-reactivity relative to the other predicted antigenic gp120 peptides in A_UG06c (Figure 15) (34, 73). This finding was unexpected given the apparently high sequence variability that is observed in this region of the envelope gene (12). An investigation of the seroreactivity of the putative PND in clones from the divergent HIV-1 clades provided further indications of antigenic conservation in the V3 region (73). The E2[V3] peptides from A_UG06c, B_RT3.12,

C_UG045, D_UG044, E_CM244 and F_RMA shared reactivities with about seventy three percent of the individual Ugandan serum samples (Table 3) (34, 73). Thus no subtype-specific antigenic phenotype could be discerned with this set of peptides and this serum panel. Furthermore, competitive ELISA (Figure 21) indicated that several of the variant peptides from clones in subtypes A through F may express conserved specificities in the PND (34, 35, 73). For instance, the patterns of sero-reactivity observed with the V3 peptides from C_UG045 and F_RMA are very similar (Table 3) (35, 73). Broad reactivities have also been previously reported for V3 peptides from certain group B isolates when tested against serum specimens from Uganda (34, 35, 162, 163) and other African communities (164-166). However, the available sequence data indicates that isolates from the subtypes B and C are not prevalent in Uganda (12). Instead, the genetic clades A and D are apparently highly prevalent in this country (12, 32, 34, 35, 163, 166). Recent attempts to relate HIV-1 serotypes to genotypes using V3 peptides has indicated the PND sequences in clones from subtypes A and C may be serologically indistinguishable (72, 166, 167). Further, phenetic analyses of the V3 sequences has suggested that this region is well-conserved in the otherwise divergent A and C subtypes of gp120 (168). Similar analyses have shown that the V3 sequences in some subtype B and D clones may also be serologically related (72, 166). The conservation of critical amino acid residues comprising and flanking the V3 loop apex may then explain the serological cross-reactivity observed among the PND sequences derived from the divergent clades (73, 167).

Data from this study indicated that the highly reactive E2[V3] peptides all contained the V3 loop apical residues, GPGR (A_UG06c and B_RT3.12) or GPGQ (C_UG045, D_UG044, E_CM244 and F_RMA) (34, 73). These tetrapeptides have been observed in this position for the majority of the HIV-1 isolates sequenced to date (12). ELISA conducted with overlapping V3 peptides from B_RT3.12 or C_UG045, displayed similar trends and intensities of reactivity with a serum panel obtained from Ugandan

donors (Figure 20). The region of overlap comprised the IGPG(R/Q)AFY residues. These data support recent reports indicating that the critical residues providing the specificity of V3 reactivity with diverse sera may be located at the cap of the loop, and may comprise a common epitope (167, 169). Secondary structure analysis of the V3 apical residues also indicated that the GPGR and GPGQ residues display comparably high probabilities of assuming β-turn conformations (Figure 17) (34). In immunological terms this finding is important since this conformation has been observed to contribute to the antigenicity of proteins (117). The R→Q substitution also occurs most frequently at this position in the HIV-1 isolates sequenced to date (12). NMR solution structure coordinates for a Thai isolate which expresses GPGQ at the cap of the V3 loop, has yielded molecular coordinates for these residues which are in the range for the antigenic β-turns (170).

These findings may not be unexpected since the biological function of the V3 loop in establishing infection may also rely on the presence of a conserved conformation in this region (171-174). A tryptic cleavage site has been identified at position i + 3, in the GPGR$^{\downarrow}$AF hexapeptide (175). Cleavage at this site has been proposed to be necessary for a conformational change to occur in gp120, allowing fusion of the viral envelope and the target cell plasma membrane to occur (173, 174). Neutralizing antibodies which bind to this region of the V3 loop have been observed to block proteolytic cleavage of the loop by thrombin (176). Thus the presence of conserved residues and/or conformations may be necessary to assure virus infectivity. However, it should be noted that some viruses may have evolved substitutions in other regions of gp120 that compensate for highly variant V3 loop sequences, thus allowing for infectivity (73). For instance, one of the isolates characterized in this study, D_UG23c, encodes a highly divergent V3 loop, but is still capable of establishing infection in PBLs and replicates to high titers (31, 32).

Although the presence of a type II β-turn in the V3 peptides usually corresponded quite well with serological reactivity, this factor alone cannot explain the antigenicity profiles observed for the divergent V3 peptides. For instance, the New York serum

samples analyzed herein displayed much more restricted V3 reactivity when compared to that observed for the Ugandan serum samples (Table 3) (34, 73). The antibodies from the North American donors reacted almost exclusively with the peptide from the subtype B clone, B_RT3.12. This limited cross-reactivity may be due to several factors: (a) the lower degree of genetic variation observed among the North American HIV-1 isolates (12); (b) the variable immune responses observed for different populations (71, 138); (c) the shorter duration of the epidemic in North America; and (d) a strong "founder" virus (HIV-1_{MN} like) effect in North America (168). Similar explanations have been proposed to account for the relatively limited sequence variation observed for the HIV-1 isolates prevalent in Thailand (168). In Thailand it has been proposed that viral genotypes may predict their neutralization serotype (151). Phylogenetic analyses have however suggested that HIV-1 infections have occurred in Africa over a longer period of time (12). This may have permitted the expansion of the immune response repertoire to the prevalent isolates.

Structural analysis of the V3 peptide complexed with mAb 59.1 provided a possible explanation for the disparate antibody reactivities observed for the Ugandan and North American sera. The highly variable flanking residues of the V3 loop do not contribute significantly to the interaction with this antibody (89). All of the hydrogen bonds and salt-bridge contacts, and 59 of the 62 van der Waals interactions observed, involve only the GPGRAF residues at the cap of the loop. Thus, the Fab in this case can make similar interactions with many otherwise variant V3 loop sequences (89, 149). A comparison of the epitopes recognized by mAb 59.1 (GPGRAFY) or by the type-specific antibody, IgG 50.1 (RIQRGPG) has also indicated that the backbone conformation of the PND residues may be highly conserved (89). These two monoclonal antibodies show low sequence identities between their CDR loops (46%); and the shapes of the Fab binding sites are completely different. Thus the epitopes recognized by the antibodies are quite different, even though they overlap in the cap region of the V3 loop. However, superimposition of the molecular coordinates of the five overlapping V3 residues, RIGPG,

indicated that the peptides have almost identical backbone structures (89). Appending the molecular coordinates of the PND residues in B_MN and C_UG045 (Figure 25) similarly indicated that the backbone structure is highly conserved in the homologous peptides (73). However, although the R→Q substitution resulted in only slightly different overall molecular conformations for the two peptides, the replacement of Arg may not permit critical side chain interactions to occur with the CDR L3 region of the antibody (Figure 25) (73). These findings may partly explain the lack of reactivity of the V3 peptides comprising GPGQ in the cap (C_UG045, D_UG044, E_CM422 and F_RMA) with mAb 59.1 or with the polyclonal sera from the New York donors.

It then appears likely that there may be two distinct but overlapping epitopes in the PND region of antigenic V3 peptides. The reactivity of the North American sera from is mimicked quite well by mAb 59.1 (Table 3 and Figure 22). Thus the dominant antigenic specificity presented by the PND in subtype B isolates also comprises an epitope(s) which requires at least several residues within the GPGRAF hexapeptide. R^{322} is apparently the most critical residue involved in the recognition of the PND by antibodies from individuals infected with subtype B isolates (73). Since R^{322} is most often found in this position in the V3 loop from the subtype B virus, the North American sera display subtype-specific ELISA reactivity. However, the specificity recognized by the broadly reactive Ugandan serum samples apparently comprises at least the IGPG residues. The presence of this tetrapeptide in a common epitope would explain the antigenic cross-reactivity observed among the otherwise divergent V3 peptides when tested with the African sera. Of greater significance is the implication that candidate vaccines based on the V3 peptides in subtype B isolates are unlikely to provide protection against the divergent subtypes of HIV-1 (73).

Substitutions at critical positions in the cap and flanking sequences in the V3 loop may significantly influence the specificities recognized by the neutralizing antibody repertoire directed at the PND (73). The analyses conducted in this study indicated a possible basis for the lack of reactivity by certain V3 peptides. The GLGQ, GQGQ, or

GPWG tetrapeptides expressed in the V3 loop of D_UG23c, D_UG042 and B_RT1, respectively, were much less antigenic than the peptides comprising GPGR and GPGQ in the same region (34). The V3 loop sequences available in the HIV-1 repository (12) indicate that isolates expressing GLGQ and GQGQ, are fairly prevalent in the African localities. However, these V3 peptides showed limited reactivity with any of the serum samples analyzed (Table 3) (34). This data is consistent with the evidence that GLGQ, GQGQ and GPWG, show low probabilities of forming β-turns (Figure 17). The homology models of the tertiary structures generated for D_UG23c and D_UG042 also indicated that substitution of proline at i + 1 (P→L or P→Q), results in markedly different overall molecular conformations for these peptides (Figure 24). The side chains in L^{320} or Q^{320} may prevent interaction with antibodies directed at the apex of the V3 cap (Figure 24) (73). Interestingly, replacement of L^{320} with proline enhances antibody binding to the V3 peptide in D_UG23c, by at least 10-fold (177, 178). This substitution may restore the β-turn secondary structure to the apical residues, thus allowing antibody binding. It is thus unlikely that the PND exists in the V3 region in the highly variant clones, B_RT1, D_UG23c and D_UG042.

It has been reported that the side chains in amino acids in divergent peptides significantly influences their ability to bind antibodies (179, 180). This study has confirmed these reports for the PND in HIV-1 gp120. Further studies will be necessary to determine whether the structural conformations of the V3 peptides described herein (73) and in other studies (88-89, 170) represents the conformation of this region when bound to antibody. It is, however, unlikely that the conformation of the free peptide is the same when bound to antibody, since ligand/receptor interactions usually involve an induced fit (180).

Antibodies directed at gp120 may also bind to conformational, discontinuous or disperse epitope(s) which are quite distinct from the continuous (linear) sequences defined in this study. Specifically, antibodies directed at the conformational CD4 binding site have been shown to recognize a discontinuous epitope (94-97). Recombinant gp120 molecules

encoded by the divergent HIV-1 clones were all capable of binding antibody from infected individuals (Figure 18) (125). Contributions to antibody binding by the V3 peptides from B_RT3, C_UG045 and D_UG044 ranged from 11% to 58% (Figure 19). The virus neutralization assay conducted herein (Table 2) also indicated the presence of alternative epitopes in gp120. Broad reactivity was observed for several serum samples in neutralization assay conducted with A_UG06c, B_IIIB and D_UG23c (31, 32). The finding that these isolates display highly divergent V3 loop sequences, but were all neutralized by a subset of the human serum antibodies is consistent with the presence of alternative or more conserved epitopes in gp120. Further, the negligible ELISA activity observed for the V3 peptide from D_UG23c (Table 3) makes it unlikely that the observed neutralization of this isolate was due to antibody binding in this region (32). Xie et. al. have also reported that immunization of mice with the ENV plasmid CMV/D_UG23c, does not elicit antibody that is detectable with V3 peptides (125). Thus while the V3 loop apparently binds a substantial proportion of the antibodies elicited in a natural infection (146, 181), there is a significant contribution by alternative immunogenic epitopes in the neutralization of HIV-1. It is feasible that a portion of the antibody reactivity observed in the neutralization assay is due to the immune response generated against the CD4-binding site, or other linear or conformational determinants.

An additional source of caution in the interpretation of the data presented herein is the impact of gp120 glycosylation on antibody response. Both O- and N-linked glycosylation have been reported for the residues comprising gp120 (182, 183). Glycosylation may result in the blocking of antibody accessibility to some of the predicted residues (184, 185). Oligomeric gp120 has also been proposed as the form in which HIV-1 binds to the CD4 molecule in natural infections (139, 186). It is quite possible that linear epitopes are more exposed in the monomeric, rather than in the oligomeric molecules of gp120 (139, 141). Distinct tertiary and quaternary structures may thus provoke unique patterns of the immune response. Accordingly, the reliability of the predictions and

serological data presented in this study may be subject to antigenic conformations that generate distinct immunogenic structures (140). Nevertheless, this report has indicated that a number of antigenic structures in gp120 are well conserved in the divergent clades of HIV-1 (34, 35, 41). These findings may be of significance in the continuing search for a broadly reactive vaccine candidate.

Appendices:

Appendix 1A. (i) Prediction of antigenic residues in gp120 from A_UG06c according to SURFACE PLOT. Residues with a high probability of surface exposure (> 60%) are shown as surface regions. Starred residues include those with intermediate (25%) probabilities of surface exposure. (ii) Amino acid residues analyzed for surface probabilities in A_UG06c.

(i)

Values: Composite(60%)
File: UG06C

Surface Region	Interior Region
	1- 27
28- 30	
	31- 51 *
52- 55	
	56- 67
68- 68	
	69- 99 *
100- 100	
102- 102	
	103- 110
111- 114	
	115- 145 *
146- 155	
	156- 173 *
174- 174	
	175- 200 *
201- 209	
213- 217	
219- 221	
225- 226	
	227- 241
242- 243	
	244- 266 *

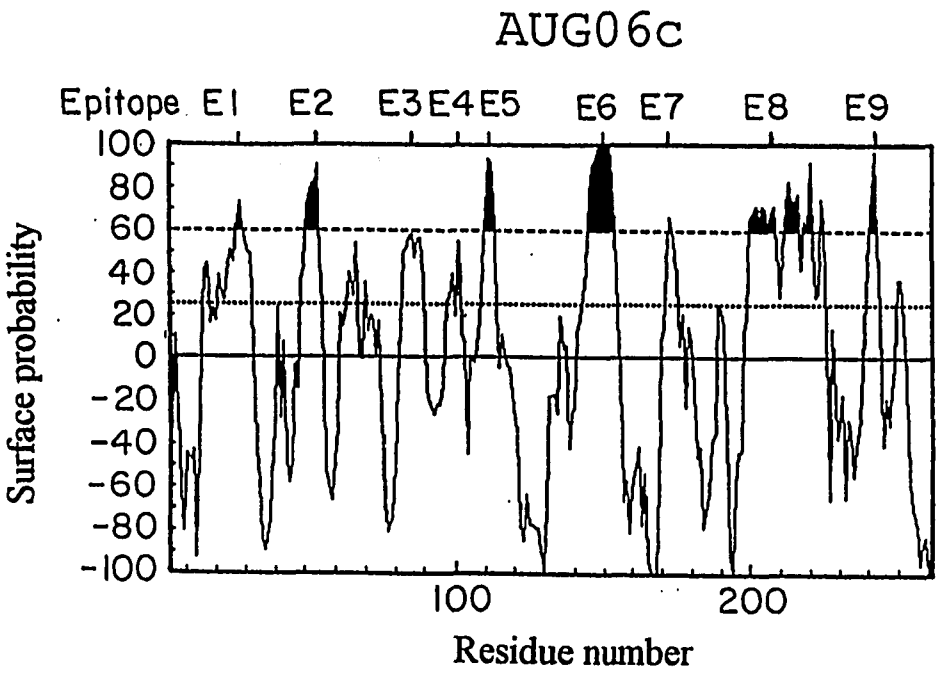
Values: Composite(25%)
File: UG06C

Surface Site No	Residues
1	17- 18
2	22- 33
3	50- 57
4	64- 64
5	66- 68
6	71- 71
7	82- 91
8	98- 104
9	110- 115
10	135- 135
11	144- 156
12	173- 178
13	180- 180
14	191- 191
15	200- 227
16	229- 229
17	241- 244
18	251- 252
19	254- 254

(ii) File: UG06C

```
                    5                  10                  15
HIS GLY ILE LYS PRO VAL VAL SER THR GLN LEU LEU LEU ASN GLY
                   20                  25                  30
SER LEU ALA GLU LYS GLU VAL LYS ILE ARG SER GLU ASN ILE SER
                   35                  40                  45
ASP ASN ALA LYS THR ILE ILE VAL GLN LEU THR LYS PRO VAL THR
                   50                  55                  60
ILE ASN CYS THR ARG PRO TYR LYS LYS VAL ARG ARG ARG ILE HIS
                   65                  70                  75
ILE GLY PRO GLY ARG SER PHE TYR THR SER ASN LEU GLY ASP ILE
                   80                  85                  90
ARG GLN ALA TYR CYS ASN VAL SER GLY SER GLN TRP ASN LYS THR
                   95                 100                 105
LEU GLN GLN VAL VAL THR LYS LEU ARG GLU TYR TRP ASN THR THR
                  110                 115                 120
ILE ASN PHE SER LYS PRO SER GLY GLY ASP LEU GLU ILE THR THR
                  125                 130                 135
HIS SER PHE ASN CYS VAL GLY GLU PHE PHE TYR CYS ASN THR SER
                  140                 145                 150
GLY LEU PHE ASN SER SER TRP LYS ILE GLY SER GLN PRO ASN SER
                  155                 160                 165
LYS GLU SER ASN GLY ASN ILE THR LEU PRO CYS ARG ILE ARG GLN
                  170                 175                 180
ILE ILE ARG ILE TRP GLN ARG THR GLY GLN ALA THR TYR ALA PRO
                  185                 190                 195
PRO ILE GLN GLY VAL ILE ARG PHE GLU SER ASN ILE THR GLY LEU
                  200                 205                 210
LEU LEU THR ARG ASP GLY GLY VAL ASN SER THR GLU GLU THR PHE
                  215                 220                 225
ARG PRO GLY GLY GLY ASP MET ARG ASP ASN TRP ARG SER GLU LEU
                  230                 235                 240
TYR LYS TYR LYS VAL VAL LYS ILE GLU PRO LEU GLY VAL ALA PRO
                  245                 250                 255
SER ARG ALA LYS ARG ARG VAL VAL TRP ARG GLU LYS ARG ALA VAL
                  260                 265                 270
VAL GLU ILE GLY ALA VAL PHE LEU GLY PHE LEU
```

Appendix 1B. Prediction of antigenic residues in A_UG06c according to POLAR TRIPEPTIDE. Symbols: areas under the peaks represent residues with at least 60% probability of surface exposure; (....) residues with at least 25% probability of surface exposure; and (-) residues with a mean probability of exposure.



Appendix 1C. Prediction of antigenic residues in A_UG06c according to PEPTIDE STRUCTURE in GCG.

Column headings: GlycoS: potential N-linked glycosylation site; HyPhil: Hydrophilicity (Kyte-Doolittle) averaged over a window of 7; SurfPr: Surface Probability according to Emini; FlexPr: Chain Flexibility according to Karplus-Schulz; CF-Pred: Secondary Structure according to Chou-Fasman; GORPred: Secondary Structure according to Garnier-Osguthorpe-Robson; AI-Index: Antigenicity Index according to Jameson-Wolf.

Pos	AA	GlycoS	HyPhil	SurfPr	FlexPr	CF-Pred	GORPred	AI-Index
16	S	.	-0.325	0.724	1.000	H	H	-0.150
17	L	.	0.520	1.133	1.000	H	H	0.900
18	A	.	1.017	1.535	1.000	H	H	0.900
19	E	.	0.271	0.850	1.000	H	H	0.450
20	K	.	0.714	2.062	1.029	H	H	0.900
21	E	.	0.614	1.431	1.031	H	H	0.900
22	V	.	1.514	1.618	1.029	H	H	0.900
23	K	.	1.129	1.084	1.036	H	H	0.900
24	I	.	1.071	1.084	1.043	H	H	0.900
25	R	.	1.071	2.349	1.057	H	H	0.900
26	S	.	1.029	0.823	1.070	H	H	0.750
27	E	.	0.586	1.574	1.063	H	H	0.900
28	N	G	1.729	1.342	1.050	.	.	0.900
29	I	.	1.586	1.611	1.037	.	.	0.900
30	S	.	1.214	0.940	1.028	t	.	0.950
31	D	.	1.271	1.168	1.029	t	T	1.500
32	N	.	0.871	2.406	1.035	t	T	1.500
33	A	.	0.871	1.258	1.033	.	.	0.900
34	K	.	0.114	0.528	1.019	.	.	0.450
35	T	.	-0.986	0.244	0.993	B	B	-0.600
36	I	.	-0.986	0.418	0.955	B	B	-0.600
37	I	.	-1.271	0.172	0.929	B	B	-0.600
38	V	.	-1.729	0.172	0.925	B	B	-0.600
39	Q	.	-1.271	0.492	0.941	B	B	-0.600
40	L	.	-0.400	1.084	0.980	B	B	-0.450
41	T	.	-0.357	1.084	1.015	.	B	0.000
42	K	.	0.343	0.904	1.031	.	.	0.450
43	P	.	-0.800	0.768	1.032	.	T	-0.050
44	V	.	0.243	0.856	1.010	B	T	0.850
45	T	.	-0.214	0.229	0.986	B	B	-0.300
46	I	.	-0.671	0.214	0.976	B	B	-0.600
47	N	G	-0.257	0.565	0.975	B	B	-0.300
48	C	.	0.571	0.605	0.993	B	B	0.600
49	T	.	0.657	1.353	1.012	B	B	0.900
50	R	.	1.857	1.683	1.026	B	.	0.900
51	P	.	1.914	6.279	1.040	T	T	1.700
52	Y	.	1.671	3.229	1.039	T	T	1.700
53	K	.	2.214	3.229	1.041	h	T	1.300
54	K	.	2.214	4.090	1.039	h	T	1.300
55	V	.	2.629	5.113	1.030	h	B	0.900
56	R	.	1.800	1.792	1.023	h	B	0.900
57	R	.	1.700	1.219	1.003	h	B	0.900
58	R	.	0.500	1.152	0.976	h	B	0.450
59	I	.	1.157	0.582	0.954	h	B	0.600

60	H	.	0.743	0.459	0.948	.	B	0.600
61	I	.	0.157	0.232	0.965	.	.	0.300
62	G	.	0.157	0.649	1.001	.	.	0.450
63	P	.	0.914	0.639	1.036	T	.	1.150
64	G	.	0.057	0.789	1.052	T	T	1.250
65	R	.	0.886	1.249	1.039	b	T	1.300
66	S	.	0.929	1.166	1.018	b	.	0.900
67	F	.	0.814	1.579	1.004	b	T	1.300
68	Y	.	1.257	1.296	1.005	b	T	1.300
69	T	.	0.071	0.798	1.022	b	T	0.850
70	S	.	0.014	0.912	1.037	T	.	0.850
71	N	.	0.914	0.972	1.040	T	T	1.550
72	L	.	0.086	0.472	1.036	.	T	0.850
73	G	.	0.629	0.690	1.034	.	T	1.150
74	D	.	1.014	0.743	1.032	.	T	1.150
75	I	.	0.257	0.910	1.026	B	B	0.450
76	R	.	0.986	1.441	1.009	B	B	0.900
77	Q	.	0.571	0.463	0.980	B	B	0.600
78	A	.	0.571	1.061	0.945	B	B	0.750
79	Y	.	0.614	0.402	0.922	B	B	0.600
80	C	.	0.086	0.311	0.922	B	B	0.300
81	N	G	-0.357	0.305	0.947	B	T	0.100
82	V	.	0.014	0.261	0.994	B	T	0.700
83	S	.	0.329	0.842	1.042	T	T	1.250
84	G	.	0.814	0.551	1.068	T	T	1.550
85	S	.	0.814	1.193	1.070	t	.	1.100
86	Q	.	1.971	1.780	1.052	B	T	1.300
87	W	.	1.957	2.596	1.032	B	T	1.300
88	N	G	1.357	1.598	1.023	B	.	0.900
89	K	.	1.743	1.598	1.024	B	.	0.900
90	T	.	1.743	2.632	1.025	B	.	0.900
91	L	.	1.014	1.215	1.018	B	.	0.900
92	Q	.	-0.086	0.451	1.005	B	B	-0.150
93	Q	.	-0.543	0.451	0.992	B	B	-0.600
94	V	.	-0.086	1.093	0.986	B	B	-0.150
95	V	.	-0.086	0.521	0.991	B	B	-0.300
96	T	.	0.057	0.589	1.005	B	B	0.450
97	K	.	0.057	1.374	1.016	.	B	0.600
98	L	.	0.843	2.900	1.014	.	B	0.900
99	R	.	1.571	2.113	1.007	.	T	1.300
100	E	.	1.971	1.699	0.992	.	T	1.150
101	Y	.	1.514	2.973	0.980	.	T	1.150
102	W	.	2.157	2.191	0.986	B	T	1.150
103	N	G	0.871	0.887	1.000	B	.	0.600
104	T	.	0.871	0.910	1.010	B	T	1.150
105	T	.	0.286	0.749	1.009	B	T	0.850
106	I	.	0.271	0.625	1.001	B	T	0.850
107	N	G	0.329	0.865	0.993	B	T	0.700
108	F	.	0.457	0.927	0.997	B	T	0.700
109	S	.	0.471	1.773	1.025	.	.	0.600
110	K	.	1.171	1.091	1.059	.	.	0.900
111	P	.	0.729	1.247	1.091	T	.	1.300
112	S	.	1.629	1.554	1.117	T	.	1.300
113	G	.	0.971	0.641	1.116	T	.	1.150
114	G	.	0.914	0.718	1.095	T	.	1.150
115	D	.	0.043	0.375	1.063	t	.	0.650
116	L	.	0.029	0.547	1.026	.	B	0.450
117	E	.	0.071	0.798	1.007	.	B	0.450
118	I	.	0.471	0.650	1.004	B	B	0.450
119	T	.	0.086	1.057	1.005	B	B	0.600

120	T	.	0.229	0.528	1.001	B	T	0.850
121	H	.	0.229	1.212	0.984	B	T	0.850
122	S	.	0.514	0.450	0.957	B	.	0.600
123	F	.	-0.186	0.232	0.936	B	T	0.100
124	N	.	-0.229	0.168	0.929	B	T	0.100
125	C	.	-0.186	0.218	0.937	B	T	0.100
126	V	.	-0.700	0.218	0.953	B	T	-0.200
127	G	.	-0.700	0.117	0.968	.	T	-0.200
128	E	.	-1.014	0.343	0.963	.	T	-0.200
129	F	.	-1.014	0.247	0.941	B	T	-0.200
130	F	.	0.086	0.402	0.924	B	T	0.700
131	Y	.	0.129	0.335	0.917	B	T	0.700
132	C	.	-0.257	0.519	0.943	B	T	0.100
133	N	G	0.200	0.593	0.990	B	T	0.700
134	T	.	0.057	0.312	1.028	B	T	0.850
135	S	.	-0.529	0.504	1.047	T	T	0.350
136	G	.	0.329	0.504	1.034	T	T	1.250
137	L	.	-0.057	0.468	1.019	.	.	-0.150
138	F	.	-0.043	0.468	1.012	.	.	-0.150
139	N	G	-0.029	0.497	1.016	.	.	-0.150
140	S	.	0.471	1.205	1.033	T	.	1.000
141	S	.	0.371	0.976	1.032	T	T	1.250
142	W	.	0.829	0.601	1.025	t	T	1.350
143	K	.	0.443	0.601	1.033	.	T	0.850
144	I	.	0.829	0.776	1.044	.	T	1.150
145	G	.	0.943	1.141	1.069	.	T	1.300
146	S	.	1.314	0.918	1.099	.	.	0.750
147	Q	.	0.871	1.754	1.114	.	.	0.900
148	P	.	2.071	3.546	1.117	T	.	1.300
149	N	.	2.514	4.582	1.120	T	.	1.300
150	S	.	2.514	3.546	1.121	t	.	1.100
151	K	.	2.514	3.687	1.119	t	.	1.100
152	E	.	2.343	2.269	1.125	t	T	1.500
153	S	.	2.343	2.723	1.122	T	T	1.700
154	N	.	1.586	0.954	1.106	T	T	1.550
155	G	.	1.129	0.795	1.082	T	T	1.550
156	N	G	0.086	0.489	1.043	t	T	1.050
157	I	.	0.200	0.471	1.006	B	T	0.850
158	T	.	-0.657	0.255	0.976	B	.	-0.600
159	L	.	-0.071	0.310	0.956	B	.	-0.300
160	P	.	-1.214	0.310	0.949	B	.	-0.600
161	C	.	0.071	0.421	0.952	B	B	0.300
162	R	.	0.471	0.885	0.963	B	B	0.300
163	I	.	0.371	0.401	0.975	B	B	0.300
164	R	.	-0.500	0.525	0.978	B	B	-0.600
165	Q	.	0.500	0.525	0.966	B	B	0.300
166	I	.	-0.786	0.525	0.942	B	B	-0.600
167	I	.	-0.014	0.282	0.921	B	B	-0.300
168	R	.	-0.157	0.282	0.914	B	B	-0.300
169	I	.	-0.014	0.787	0.924	B	T	0.100
170	W	.	0.729	1.620	0.954	B	T	1.150
171	Q	.	1.429	0.819	0.997	B	.	0.600
172	R	.	1.286	2.022	1.031	B	T	1.300
173	T	.	1.671	1.943	1.056	B	T	1.300
174	G	.	1.643	1.619	1.062	B	T	1.300
175	Q	.	1.329	1.295	1.040	B	T	1.300
176	A	.	0.429	0.907	1.013	B	.	0.450
177	T	.	0.557	1.417	0.989	B	.	0.750
178	Y	.	0.729	1.265	0.983	B	.	0.750
179	A	.	-0.414	0.878	0.994	B	.	-0.600

180	P	.	0.343	1.053	1.016	.	.	0.600
181	P	.	0.300	0.665	1.033	.	T	0.850
182	I	.	-0.486	0.489	1.030	B	T	-0.050
183	Q	.	-0.871	0.222	1.015	B	B	-0.450
184	G	.	-0.457	0.281	0.988	B	B	-0.600
185	V	.	-1.086	0.347	0.956	B	B	-0.600
186	I	.	0.057	0.347	0.941	B	B	0.300
187	R	.	-0.329	0.469	0.950	B	B	-0.300
188	F	.	0.114	1.017	0.974	B	B	0.450
189	E	.	0.071	1.017	1.009	.	B	0.600
190	S	.	0.814	0.749	1.039	t	.	0.950
191	N	G	0.229	0.857	1.043	t	T	1.050
192	I	.	0.086	0.408	1.037	B	T	0.850
193	T	.	-0.957	0.251	1.023	B	.	-0.450
194	G	.	-1.614	0.129	1.001	B	.	-0.450
195	L	.	-2.014	0.265	0.988	B	B	-0.600
196	L	.	-0.729	0.360	0.983	B	B	-0.600
197	L	.	-0.329	0.607	0.990	B	B	-0.300
198	T	.	-0.329	0.728	1.013	B	B	-0.150
199	R	.	0.271	0.874	1.036	T	T	1.250
200	D	.	0.214	0.787	1.050	T	T	1.250
201	G	.	1.257	0.876	1.056	T	T	1.550
202	G	.	1.271	0.600	1.052	t	.	0.950
203	V	.	0.729	0.518	1.050	t	.	0.950
204	N	G	0.729	0.907	1.059	t	.	0.950
205	S	.	1.171	1.587	1.075	t	.	1.100
206	T	.	1.214	3.086	1.082	t	.	1.100
207	E	.	1.414	1.662	1.078	.	B	0.900
208	E	.	1.557	2.429	1.063	.	B	0.900
209	T	.	1.671	2.602	1.040	.	B	0.900
210	F	.	1.629	1.487	1.031	.	B	0.900
211	R	.	1.186	0.850	1.043	.	.	0.750
212	P	.	0.743	0.583	1.067	T	T	1.550
213	G	.	1.143	1.124	1.095	T	T	1.700
214	G	.	1.271	0.568	1.105	T	.	1.150
215	G	.	1.271	0.719	1.095	t	.	0.950
216	D	.	1.543	1.214	1.071	t	.	1.100
217	M	.	1.986	1.972	1.040	t	.	1.100
218	R	.	2.057	2.095	1.019	t	.	1.100
219	D	.	2.643	2.457	1.006	T	T	1.700
220	N	.	2.257	3.328	1.008	T	T	1.700
221	W	.	3.029	2.942	1.017	.	.	0.900
222	R	.	1.843	1.453	1.030	.	.	0.900
223	S	.	1.529	1.416	1.000	.	.	0.900
224	E	.	1.200	1.721	1.000	.	T	1.300
225	L	.	1.260	1.123	1.000	.	T	1.300
226	Y	.	0.450	1.071	1.000	.	T	1.000

Bibliography

1. Levy JA. 1993. Pathogenesis of human immunodeficiency virus infection. Microbiol. Rev. 57: 183-289.

2. Bour S, Geleziunas R and Wainberg MA. 1995. The human immunodeficiency virus type 1 (HIV-1) CD4 receptor and its central role in promotion of HIV-1 infection. Microbiol. Rev. 59(1): 63-93.

3.Tersmette M, deGoede REY, Al BJ, Winkel IN, Grutes RA, Cuypers HT, Huisman HG and Miedema F. 1988. Differential syncytium-inducing capacity of human immunodeficiency virus isolates: frequent detection of syncytium-inducing isolates in patients with AIDS and AIDS-related complex. J. Virol. 62: 2026-2032.

4. Koot M, Keet IPM, Vos AHV, deGoede REY, Roos M, Coutinho RA, Miedema F and Tersmette M. 1992. Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4+ cell depletion and progression to AIDS. Ann. Intern. Med. 118: 681-688.

5. Von Briesen H, Andersen R and Rubsamen-Waigmann H. 1990. Systematic classification of HIV biological subtypes on lymphocytes and monocytes/macrophages. Virology. 178: 597-602.

6. Zhu T, Mo H, Wang N, Nam DS, Cao Y, Koup RA and Ho DD. 1993. Genotypic and phenotypic characterization of HIV-1 in patients with primary infection. Science. 261: 1179-1181.

7. WHO Network for HIV Isolation and Characterization. 1994. HIV-1 variation in World Health Organization-sponsored vaccine evaluation sites: Genetic screening, sequence analysis and preliminary biological characterization of selected viral strains. AIDS Res. Hum. Retroviruses. 10: 1327-1343.

8. Benditt J. 1993. AIDS: the unanswered questions. Science. 260: 1253-1271.

9. Fields BN and Knipe DM. 1991. Fundamental Virology. 2nd Ed. Raven Press, New York. pp 645-723.

10. White DO and Fenner FJ. 1994. Medical Virology (Fourth Ed.). Academic Press, San Diego, CA. pp 532-560.

11. Cheng-Mayer C, Weiss C, Seto D, and Levy JA. 1989. Isolates of human immunodeficiency virus type 1 from the brain may constitute a special group of the AIDS virus PNAS (USA). 80: 8575-8579.

12. Myers G, Korber B, Smith RF, Berzofsky JA and Pavlakis GN. (Eds.). 1992-1994. In Human Retroviruses and AIDS. A compilation of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, NM.

13. Hahn B, Robertson DL, Louwagie J, McCutchan FE and Sharp PM. 1995. Genetic diversity and recombination in HIV. In Molecular Pathogenesis of HIV-1. Keystone Symposia, CO. J. Cell. Biochem. (Suppl. 12B). Abstract No. D4-012.

14. Embertson J, Zupanic M, Ribas JL, Burke A, Racz P, Tenner-Racz K and Haase AT. 1993. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. Nature (London). 362: 359-362.

15. Bhat S, Spitalnik Sl, Gonzalez-Scarano F, and Silberberg DH. 1991. Galactosyl ceramide or a derivative is an essential component of the neural receptor for human immunodeficiency virus type 1 envelope glycoprotein gp120. PNAS (USA). 88: 7131-7134.

16. Fantini J, Cook DG, Nathanson N, Spitalnik SL and Gonzalez-Scarano F. 1993. Infection of colonic epithelial cell lines by type 1 human immunodeficiency virus is associated with cell surface expression of galactosylceramide, a potential alternative gp120 receptor PNAS (USA). 90: 2700-2704.

17. Weiss RA. 1993. How does HIV cause AIDS? Science. 260: 1273-1279.

18. Pantaleo G, Graziosi C, Demarest JF, Butini L, Montroni M, Fox CH, Orenstein JM, Kotler DP and Fauci AS. 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of infection. Nature (London). 362: 355-358.

19. Nowak R. 1991. AIDS vaccines: Key questions still unanswered. J. NIH Res. 3: 37-39.

20. Cann A. 1994. Principles of Molecular Virology. Academic Press, San Diego, CA.

21. Hahn B, Shaw G, Taylor ME, Redfield RR, Markham, Salahuddin SZ, Wong-Staal F, Gallo RC, Parks ES and Parks WD. 1986. Genetic variation in HTLV-III/LAV over time in patients with AIDS or at risk for AIDS. Science. 232: 1548-1553.

22. Fisher AG, Ensoli B, Looney D, Rose A, Gallo RC, Saag MS, Shaw BH and Wong-Staal F. 1988. Biologically diverse molecular variants within a single HIV-1 isolate. Nature. 334: 440-444.

23. Bonhoeffer S, Holmes EC and Nowak MA. 1995. Causes of HIV diversity. Nature. 376: 125.

24. Reitz MS, Wilson C, Nangle C, Gallo RC and Robert-Guroff M. 1988. Generation of a neutralization-resistant variant of HIV-1 is due to selection for a point mutation in the envelope gene. Cell. 54: 57-63.

25. Moore JP, Cao Y, Ho DD and Koup RA. 1994. Development of anti-gp120 antibody response during seroconversion to Human Immunodeficiency Virus type 1. J. Virol. 5142-5155.

26. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing W and Ho DD. 1994. Temporal association of cellular immune response with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J. Virol. 68: 4650-4655.

27. Haynes BF. 1996. Toward an understanding of the correlates of protective immunity to HIV infection. Science. 271: 324.

28. Meier U-C, Klenerman P, Griffin P, James W, Koppe B, Larder B, McMichael A and Phillips R. 1995. Cytotoxic T lymphocytes lysis inhibited by viable HIV mutants. Science. 270: 1360-1362.

29. Osmanov S, Heyward WL and Esparza J. 1994. The World Health Organization Network for HIV isolation and characterization: summary of a pilot study. AIDS Res. Hum. Retroviruses. 10(11): 1325-1326.

30. Oram JD, Downing RG, Roff M, Sewankambo N, Clegg JCS, Featherstone ASR and Booth JC. 1991. Sequence analysis of the V3 loop regions of the ENV genes of Ugandan human immuno-deficiency proviruses. AIDS Res. Hum. Retroviruses. 6: 605-614.

31. Atkin A, Pestano G, Serwadda D, Prince A, Pascual D, Sewankambo N and Boto WMO. 1993. Phylogenetic and serological characterization of two Ugandan HIV-1 isolates. AIDS Res. Hum. Retroviruses. 9: 351-356.

32. Pestano G, Prince A, Guyden J, Ntambi J, Atkin A, and Boto WO. 1993. Independent divergences in the CD4 binding site and V3 loop encoded in two seroprevalent Ugandan HIV-1 isolates. J Acqui. Immun. Defic. Syn. 6: 872-888.

33. Pestano G, Atkin A, Sewankambo N, Prince A, Serwadda D and Boto, WO. 1992. Molecular cloning and analysis of cDNAs encoding the V3 loops in two seroprevalent Ugandan HIV-1 isolates. Fifth Annual Meeting of the NCVDG for AIDS. Abstract No. 19.

34. Pestano GA, Hosford KS, Riley J, Spira AI, Xie J-M, Sewankambo N, Brown L, Ho DD and Boto WMO. 1995. Sero-reactivity of analogous and distinct antigenic epitopes in glycoprotein gp120 expressed in HIV-1 subtypes A, B, C, and D. AIDS Res. Hum. Retroviruses. 11(5): 589-596.

35. Riley J, Pestano G, Francis C, Xie J-M, Mugyeni P, Kataaha P, Katongle-Mbidde E, Anokbonggo W and Boto WMO. 1995. Sero-prevalence of the indigenous HIV-1 subtypes A, C and D in Ugandan regional communities. Arch. Virol. 10(8): 1393-1404.

36. Nkengasong JN, Janssens W, Heyndrickx L, Frasen K, Ndumbe PM, Motte J, Leonares A, Ngolbe M, Ayuk J, Piot P and van der Groen G. 1994. Genotypic subtypes of HIV-1 in Cameroon. AIDS. 8: 1405-1412.

37. Janssens W, Heyndrickx L, Frasen K, Motte J, Peeters M, Nkengasong JN, Ndumbe PM, Delaporte E, Perret J-L, Atende C, Piot P, and Van der Groen. 1994. Genetic and phylogenetic analysis of env subtypes G and H in Central Africa. AIDS Res. Hum. Retroviruses. 10:877-879.

38. Akimbu AG, Stern TL, Zwor A, Markham PD, Calef PD, Kyari S, Saxinger WC, Gallo RC, Robert-Guroff M and Reitz MS. 1994. Subgroup G isolates from Nigeria. AIDS Res. Hum. Retroviruses. 10: 1581.

39. Pestano G, Hosford K, Pascual D, Riley J, Osada M, Prince A and Boto W. 1995. Antigenic variation in the principal neutralizing determinant from HIV-1 subtypes A through F. In UCLA/UCI Annual AIDS Symposium: Mechanisms of HIV Disease. Abstract No. 42.

40. Rusche JR, Javaherian K, McDanal C, Petro J, Lynn DL, Grimaila R, Langlois A, Gallo RC, Fischinger PJ, and Bolognesi DP. 1988. Antibodies that inhibit fusion of human immunodeficiency virus-infected cells bind a 24-amino acid sequence of the viral envelope, gp120. PNAS (USA). 85: 3198-3202.

41. Riley J, Pestano GA, Harewood K, Guyden J, Alfred L and Boto WMO. 1995. Distribution of linear antigenic epitopes on gp120 encoded in sibling clones of novel HIV-1 subtype B strains. Cell Mol. Biol. 41(Suppl. 1): 83-91.

42. McCutchan FE, Hegerich PA, Brennan TP, Phanuphak P, Singharaj P, Jugsudee A, Berman P, Gray AM, Fowler AK and Burke DS. 1992. Genetic variants of HIV-1 in Thailand. AIDS Res. Hum. Retroviruses. 8: 1887-1895.

43. Dumitrescu O, Kalish M, Kliks SC, Bandea CI, Levy J. 1994. Characterization of HIV-1 strains isolated from children in Romania: identification of a new envelope subtype. J Infect. Dis. 169: 281-288.

44. Potts K, Kalish ML, Lott T, Orloff G, Luo C-C, Bernard M-A, Brits-Alves C, Badaro R, Suleiman J, Ferreira O, Schochetman G, Johnson WD, Ou C-Y, Ho J-L and the Brazilian Collaborative AIDS Research Group. 1993. Genetic heterogeneity of the V3 region of the HIV-1 envelope glycoprotein in Brazil. AIDS. 7: 1191-1197.

45. Louwagie J, Delwart EL, Mullins JI, McCutchan FE, Eddy G and Burke DS. 1994. Genetic analysis of HIV-1 isolates from Brazil reveals the presence of two distinct genetic subtypes. AIDS Res. Hum. Retroviruses. 10: 561-567.

46. Morgado MG, Sabino EC, Shpear EG, Bongertz V, Brigido L, Guimaraes MDC, Castilho EA, Galvao-Castro B, Mullins JI, Hendry RM and Mayer A. 1994. V3 region polymorphisms in HIV-1 from Brazil: prevalence of subtype B strains divergent from North American/European prototype and detection of subtype F. AIDS Res. Hum. Retroviruses. 10: 569-576.

47. Vanden Haesvelde M, Cecourt J-L, De Leys RJ, Vanderbroght B, van der Groen G van Heuverswijn H and Saman E. 1994. Genomic cloning and complete sequence analysis of a highly divergent African human immunodeficiency virus isolate. J. Virol. 68: 1586-1596.

48. Nkengasong JN, Peeters M, vanden Haesvelde M, et. al. 1993. Antigenic evidence of the presence of the aberrant HIV-1ANT70 virus in Cameroon and Gabon [letter]. AIDS. 7: 1536-1538.

49. Salk J, Bretscher PA, Salk P, Clerici M and Shearer G. 1993. Strategy for prophylatic vaccination against HIV. Science. 260: 1270-1272.

50. Sabin A, et al. 1988. Improbability of effective vaccination against human immunodeficiency virus because of its intracellular transmission and rectal portal of entry. PNAS (USA). 85: 6944-6948.

51. Prince A, Horowitz B, Baker L, Shulman RW, et al. 1988. Failure of an HIV immune globulin to protect chimpanzees against experimental challenge with HIV. PNAS (USA). 85: 6944-6948.

52. Koff WC and Hoth DF. 1988. Development and testing of AIDS vaccines. Science. 241: 426-432.

53. Fauci AS, Gallo RC, Koenig S, Salk J and Purcell RH. 1989. Development and evaluation of a vaccine for human immunodeficiency virus (HIV) infection. Ann. Int. Med. 110(5): 373-385.

54. Kurth R, Binninger D, Ennen J, Denner J, Hartung S and Norley S. 1991. The quest for and AIDS vaccine: the state of the art and current challenges. AIDS Res. Hum. Retroviruses. 7(5): 425-433.

55. Derosiers RC, Wyand MS, Kodama T, Ringler DJ, Arthur LO, Sehgal PK, Letvin NL, King NW and Daniel MD. 1989. Vaccine protection against simian immunodeficiency virus infection. PNAS (USA). 86: 6353-6357.

56. Gardner M and Hu S-L. 1991. SIV vaccines, 1991:a year in review. AIDS. 5:115-127.

57. Daniel M, Kirchloff F, Czajak SC, Sehgal PK, et al. 1992. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. Science. 258: 1938-1941.

58. Haynes BF, Torres JV, Langlois AJ, Bolognesi DP, Gardner MB, Palker TJ, Scearce RM, Jones DM, Moody MA, McDanal C and Matthews TJ. 1993. Induction of HIVMN neutralizing antibodies in primates using a prime-boost regimen of hybrid synthetic gp120 envelope peptides. J. Immunol. 151: 1646-1653.

59. Bourgault I, Chirat F, Tartar A, Levy JP, Guillet JG and Venet A. 1994. Simian immunodeficiency virus as a model for vaccination against HIV. J. Immunol. 2530-2357.

60. Ruprecht R, Baba TW, Joeng YS, Bronson R, Penninck D and Greene MF. 1995. Differential pathogenicity of multiply deleted SIV in adult and neonatal rhesus monkeys: implications for live-attenuated viurs vaccine strategies. In Molecular Pathogenesis of HIV-1. Keystone Symposia, CO. J. Cell. Biochem (Suppl. 21B). Abstract D4-011.

61. Prince A, Horowitz B, et al. 1990. Apparent prevention of HIV infection by HIV immmunoglobulin given prior to low-dose HIV challenge. Vaccines. 90: 347-351.

62. Prince A, Reesink H, Pascual D, et al. 1991. Prevention of HIV infection by passive immunization with HIV immunoglobulin. AIDS Res. Hum Retroviruses. 7: 971-973.

63. Goedert J, Mendez H, Drummond J, Robert-Guroff M, et al. 1989. Mother to infant transmission of Human immunodeficiency virus type-1: association with prematurity and low anti-gp120. Lancet. 2: 1351-1354.

64. Rossi P, Moschese V, Broliden P, Fundaro C, et al. 1989. Presence of maternal antibodies to human immunodeficiency virus 1 envelope glycoprotein gp120 correlates with the uninfected status of children born to seropositive mothers. PNAS (USA). 86: 8055-8058.

65. Parekh BS, Shaffer N, Parr CP, Abrams E, et al. 1991. Lack of correlation between maternal antibodies to V3 loop peptides of gp120 and perinatal HIV-1 transmission. AIDS. 5: 1179-1184.

66. Halsey NA, Markham R, Wahren B, Bonloc R, et al. 1992. Lack of association between maternal antibodies to the V3 loop peptides and maternal-infant HIV-1 transmission. J. Acq. Imm. Def. Syn. 5: 153-157.

67. Berman PW, Matthews TJ, Riddle L, Champe M, Hobbs MR, Nakamura GR, Mercer J, Eastman DJ, Lucas C, Langlois AJ, et. al. 1992. Neutralization of multiple laboratory and clinical isolates of human immunodeficiency virus type 1 (HIV-1) by antisera raised against gp120 from the MN isolate of HIV-1. J. Virol. 66: 4464-4469.

68. Matthews TJ. 1994. Dilemma of neutralization resistance of HIV-1 field isolates and vaccine development. AIDS Res. Hum. Retroviruses. 10: 631-632.

69. Moore JP. 1995. HIV vaccines: back to primary school. News and Views. Nature. 376: 115.

70. Wrin T, Crawford L, Sawyer L, Weber P, Shepperd HW, Hanson CV. 1994. Neutralizing antibody responses to autologous and heterologous isolates of human immunodeficiency virus. J. Acq. Immun. Def. Syn. 7: 211-219.

71. Schrier RD, Gnann JW, Landes R, Lockshin C, Oldstone MB and Nelson JA. 1989. T cell recognition of HIV synthetic peptides in a natural infection. J. Immunol. 142: 1166-1176.

72. Moore JP, McCutchan FE, Poon S-W, Mascola J, Liu J, Cao Y and Ho DD. 1994. Exploration of antigenic variation in gp120 from clades A through F of human immunodeficiency virus type 1 by using monoclonal antibodies. J. Virol. 68(12): 8350-8364.

73. Pestano GA, Hosford KS, Lee S-K, Riley JP, Silver S and Boto WMO. 1996. Impact of sequence variation on the structural conformations and antigenicity of the PND in HIV-1. J. Virol. (submitted).

74. Oram JD, Downing RG, Roff M, Clegg JCS, Serwadda D, and Carswell M. 1990. Nucleotide sequence of a Ugandan HIV-1 provirus reveals genetic diversity from other HIV-1 isolates. AIDS Res. Hum. Retroviruses. 6(9): 1073-1078.

75. Bruce C, Clegg C, Featherstone A, Smith J and Oram J. 1993. Sequence analysis of the gp120 region of the env gene of Ugandan human immunodeficiency provirus from a single individual. AIDS Res. Hum. Retroviruses. 9(4): 357-363.

76. Albert J, Franzen L, Jansson M, Scarlatti G, Kataahaa PK, Katabira E, Mubiro F, Rydaker M, Rossi P, Petersson U and Wigzel H. 1992. Ugandan HIV-1 V3 loop sequences closely related to the US/European consensus. Virology. 190: 674-681.

77. Javaherian K, Langlois AJ, McDanal C, Ross KL, Eckler LI, Jellis C, Profy AT, Rusche JR, Bolognesi DP, Putney S, and Matthews TJ. 1989. Principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein. PNAS (USA). 86: 6768-6772.

78. Modrow S, Hahn BH, Shaw GM, Gallo RC, Wong-Staal F and Wolf, H. 1987. Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions. J. Virol. 61: 570-578.

79. Scott CF, Jr., Silver S, Profy AT, Putney SD, Langlois A, Weinhold K, and Robinson JE. 1990. Human monoclonal antibody that recognizes the V3 region of human immunodeficiency virus gp120 and neutralizes the human T-lymphotrophic virus type IIIMN strain. PNAS (USA) 87: 8597-8601.

80. Neurath AR, Strick N, Fields R and Jiang S. 1991. Peptides mimicking selected disulfide loops in HIV-1 gp120, other than V3, do not elicit virus-neutralizing antibodies. AIDS Res. Hum. Retroviruses. 7(8): 657-662.

81. Broliden P-A, von Gegerfelt A, Clapham P, Rosen J, Fenyo E-M, Wahren B and Broliden K. 1992. Identification of neutralization-inducing regions of the human immunodeficiency virus type 1 envelope glycoproteins. PNAS (USA). 89: 461-465.

82. Laman JD, Schellekens MM, Abacioglu YH, Lewis GK, Tersmette M, Fouchier AM, Langedijk JP, Claassen E and Boersma WJ. 1992. Variant-specific monoclonal and group-specific polyclonal human immunodeficiency virus type 1 neutralizing antibodies raised with synthetic peptides from the gp120 third variable domain. J. Virol. 66: 1823-1831.

83. Kenaly WR, Matthews TJ, Granfield MC, Langlois AJ, Waselefsky DM, and Petteway SR. 1989. Antibodies from human immunodeficiency virus-infected individuals bind to a short amino acid sequence that elicits neutralizing antibody response in animals. AIDS Res. Hum. Retroviruses. 5: 173-182.

84. Bradac JA and Mathieson BJ. June 1991. An epitope map of immunity to HIV-1: a roadmap for vaccine development. In International Conference on AIDS. Abstract No. M.A. 1328.

85. Conley AJ, Gorny MK, Kessler JA, Boots LJ, Ossorio-Castro M, Koenig S, Lineberger DW, Emini EA, Williams C and Zolla-Pazner. 1994. Neutralization of primary human immunodeficiency virus type 1 isolates by the broadly reactive anti-V3 monoclonal antibody, 447-53D. J. Virol. 68: 6994-7000.

86. LaRosa GJ, Davide JP, Weinhold K, et al. 1990. Conserved sequence and structural elements in the HIV-l principal neutralizing determinant. Science. 249: 933-935.

87. Catasti P, Fontenot JD, Bradbury EM and Gupta G. 1995. Local and global structural properties of the HIV-MN V3 loop. J. Biol. Chem. 270(3): 2224-2232.

88. Rini JM, Stanfield RL, Stura E, Salinas PA, Profy AT and Wilson IA. 1993. Crystal structure of a human immunodeficiency virus type 1 neutralizing antibody, 50.1, in complex with its V3 loop peptide antigen. PNAS (USA). 90: 6325-6329.

89. Ghaira J, Stura E, Stanfield R, Profy A and Wilson I. 1994. Crystal structure of the principal neutralization site of HIV-1. Science. 264: 82-85.

90. Palker TJ, Clark ME, Langlois AJ, Matthews TJ, Weinbold KJ, Randall RR, Bolognesi DP and Haynes BF. 1988. Type-specific neutralization of the human immunodeficiency virus with antibodies to env-encoded synthetic peptides. PNAS (USA). 85: 1932-1936.

91. Gorny MK, Xu J-Y, Gianakabos V, Karwowska S, Williams C, Sheppard HW, Hanson CV and Zolla-Pazner S. 1991. Production of site-selected neutralizing human monoclonal antibodies against the third variable domain of the human immunodeficiency virus type 1 envelope glycoprotein. PNAS (U.S.A.) 88: 3238-3242.

92. Ivanoff L, Looney DJ, McDanal C, Morris JF, Wong-Staal F, Langlois AJ, Petteway SR and Mathews TJ. 1991. Alteration of HIV-1 infectivity and neutralization by a single amino acid replacement in the V3 loop domain. AIDS Res. Human Retroviruses. 26: 595-603.

93. Lasky L, Nakamura G, Smith DH, Fennie C, Shimasaki C, Patzer E, Berman P, Gregory T and Capon DJ. 1987. Delination of a region of the human immunodeficiency virus type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. Cell. 50: 975-985.

94. Olshevsky U, Helseth E, Furman C, Li J, Haseltine W, and Sodorski J. 1990. Identification of individual human immunodeficiency type 1 gp120 amino acids important for CD4 receptor binding. J. Virol. 64: 5701-5707.

95. Ho DD, McKeating JA, Li XL, Moudgil T, Daar ES, Sun NC, and Robinson JE. 1991. Conformational epitope on gp120 important in CD4 binding and human immunodeficiency virus type 1 neutralization identified by a human monoclonal antibody. J. Virol. 65: 489-493.

96. Thali M, Furman C, Wahren B, Posner M, Ho DD, Robinson J and Sodroski, J. 1992. Cooperativity of neutralizing antibodies directed against the V3 and CD4 binding regions of the human immunodeficiency virus gp120 envelope glycoprotein. J. Acq. Imm. Def. Syn. 5: 591-599.

97. Thali M, Furman C, Ho DD, Robinson J, Tilley S, Pinter A and Sodroski, J. 1992. Discontinuous, conserved neutralizing epitopes overlapping the CD4-binding region of Human Immunodeficiency Virus type 1 gp120 envelope glycoprotein. J. Virol. 66: 5635-5641.

98. Thali M, Moore JP, Furman C, Charles M, Ho DD, Robinson J and Sodroski J. 1993. Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. J. Virol. 67(7): 3978-3988.

99. Nakamura GR, Byrn R, Rosenthal K, Porter JP, Hobbs MR, Riddle L, Eastman DJ, Dowbenko D, Gregory T, Fendly BM, and Berman PW. 1992. Monoclonal antibodies to

the extracellular domain of HIV-1 IIIB gp160 that neutralize infectivity, block binding to CD4, and react with diverse isolates. AIDS Res. Hum. Retroviruses. 8: 1875-1885.

100. Pinter A, Honnen WJ, Racho ME and Tilley S. 1993. A potent, neutralizing human monoclonal antibody against a unique epitope overlapping the CD4 binding site of HIV-1 gp120 that is broadly conserved across North American and African virus isolates. AIDS Res. Hum. Retroviruses. 9(10):985-996.

101. Tilley S, Honnen WJ, Racho ME, Chou T-C and Pinter A. 1992. Synergistic neutralization of HIV-1 by human monoclonal antibodies against the V3 loop and the CD4-binding site of gp120. AIDS Res. Hum. Retroviruses. 8(4): 461-467.

102. Robinson W and Mitchell W. 1990. Neutralization and enhancement of in vitro and in vivo HIV and simian immunodeficiency virus infections. AIDS. 4: 5151-5162.

103. Boto W, Brown L and Adler, W. 1991. Distinct modulatory effects of bryostatin 1 and staurosporine on the biosynthesis and expression of the HIV receptor protein (CD4) by T cells. Cell Regulation. 2: 95-103.

104. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB and Erlich K. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. 239: 487-491.

105. Chomczynski P and Sacci N. 1997. Single-step method for RNA isolation by acid guanidinium thiocyanate-phenol-cholorform extraction. Anal. Biochem. 162:156-158.

106. Innis M, Gelfand D, Sninsky, J and White, T. 1992. PCR Protocols: A guide to methods and applications. Academic Press Inc., San Diego, CA. pp 337-347.

107. Pestano G, Sewankambo N, Magnet M, Ntambi J, Serwadda D and Boto, W. 1990. Update on DNA polymerase chain reaction (PCR) in the study of AIDS: Relavance to clinical studies and molecular analysis of the HIV genome function. In 1st Annual RCMI/NIH AIDS Symposium. p9.

108. Sambrook J, Fritsch EF and Maniatis T. In Molecular Cloning. A laboratory manual. (Second Ed.). Cold Spring Harbor Laboratory Press, Plainview, NY. 1989.

109. Sanger K, Nicklen S and Coulson, AR. 1977. DNA sequencing with chain-terminating inhibitors. PNAS (USA). 74: 5463-5467.

110. Felsenstein J. 1989. PHYLIP: Phylogeny Inference Package (Version 3.2). Cladistics. 5: 164-166.

111. Kumar S, Tamura K and Nei M. 1993. MEGA: Molecular Evolutionary Genetics Analysis program package (version 1.0). The Pennsylvania State University, University State Park, Pennsylvania.

112. Parker JMR and Hodges RS. 1991. Prediction of surface and interior regions in proteins-part 1: linear tripeptide sequences identify structural boundaries in proteins. Peptide Res. 4: 347-354.

113. Devereux J, Haeberly P and Smithies, O. 1984. A comprehensive set of sequence analysis programs for the VAX. Nuc. Acids Res. 12: 387-395.

114. Wolf H, Modrow S, Motz M, Jameson BA, Hermann G and Fortsch B. 1988. An integrated family of amino acid sequence analysis programs. CABIOS. 4: 187-191.

115. Pestano G, Atkin A and Boto W. 1993. Predictions of linear antigenic peptides in gp120 sequences of two divergent Ugandan HIV-1 isolates. FASEB J. 6(4): Abstract No. 2905.

116. Hosford K, Pestano G, Riley J, Spira A, Brown L, Ho D and Boto WMO. 1994. HIV-1 field isolates from Africa and New York display analogous antigenic sites in gp120. FASEB J. 8(5): Abstract No. 4644.

117. Chou P and Fasman GD. 1978. Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzmol. 47: 45-148.

118. Sayle, R. 1994. RASMOL, version 2.5: a molecular visualization program. Glaxo Research and Development, Biomolecular Structures Group, Greenford, Middlesex U.K.

119. Richardson DC and Richardson JS. 1992. The kinemage: a tool for scientific communication. Protein Science. 1(1): 3-9.

120. Richardson, DC and Richardson, JS. 1994. Kinemages: simple macromolecular graphics for interacive teaching and publication. TIBS. 19: 135-138.

121. Garnier J and Levin JM. 1991. The protein structure code: what is its present status? CABIOS. 7(2): 133-142.

122. Garnier J. 1990. Protein structure prediction. Biochemie. 72: 513-524.

123. Merrifield R. 1963. Solid-phase peptide. The synthesis of a tetrapeptide. J. Am. Chem. Soc. 85: 2149.

124. Chapman BS, Thayer RM, Vincent KA and Haigwood NL. 1991. Effects of intron A from human cytomegalovirus (Towne strain) immediate-early gene on heterologous expression in mammalian cells. Nuc. Acids Res. 19: 3979.

125. Xie J-M, Pestano GA, Lee S-K, Samms M, Guyden J, Coico R and Boto WMO. 1996. Immunogenic potential of rgp120 from African HIV-1 subtype A. Vaccine. (in press).

126. Foecking MN and Hofsetter H. 1986. Powerful and versatile enhancer-promoter unit for mammalian expression vectors. Gene. 45: 101.

127. Crockett ML, Bebbington CR and Yarranton GT. 1991. The use of engineered E1A gene to transactivate the hCMV-MIE promoter in permanent CHO cell lines. Nuc. Acids Res. 19: 319.

128. Rhodes G, Dwaki V, Abai AM, Felgner J et.al. 1993. Injection of expression vectors containing viral genes induces cellular, humoral, and protective immunity. In Vaccines '93. Modern approaches to new vaccines including prevention of AIDS. Ginsberg HS, Brown F, Chanock RM and Lerner RA. (eds). Cold Spring Harbor Laboratory Press, Plainview, New York. 93: 137.

129. Ulmer J, Donnelly JJ, Parker SE, Rhodes GH, et. al. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science. 259: 1745.

130. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248.

131. Boto WO, Powers K and Levy D. 1984. Antigens of Dirofilaria immitis which are immunogenic in their canine host: detection by immuno-staining of protein blots with the antibodies of occult dogs. J. Immunol. 133: 975-980.

132. Wawer MJ, Serwadda D, Musgrave SD, Konde-Lule JK, Musagara M and Sewankambo NK. 1991. Dynamics of HIV-1 infection in a rural district of Uganda. BMJ. 303: 1303-06.

133. Serwadda D, Wawer MJ, Musgrave SD, Sewankambo N, Kaplan J and Gray RH. 1992. HIV risk factors in three geographic strata of rural Rakai District, Uganda. AIDS. 6: 983-989.

134. Mulder D, Nunn A, Wagner H-U, Kamali, A and Kengeya-Kayondo J. 1994. HIV-1 incidence and HIV-1 associated mortality in a rural Ugandan population chort. AIDS. 8: 87-92.

135. Nkengasong JN, Peeters M, Ndumbe PM, Janssens W, Willems B, Frasen K, Ngolle M, Piot P and van der Groen G. 1994. Cross-neutralizing antibodies to HIV-1ANT70 and HIV1IIIB in sera of African and Belgian HIV-1 infiected individuals. AIDS. 8(8): 1089-1096.

136. Berzofsky J. 1985. Intrinsic and extrinsic factors in protein antigenic structure. Science. 219: 932-940.

137. Appel JR, Pinilla C, Niman H and Houghten R. 1990. Elucidation of discontinuous linear determinants in peptides. J. Immunol. 144: 976-983.

138. Sexton J, Wilce M, Colin T, Wijffels G, Salvatore L, Feil S, Parker M, Spithill T and Morrison C. 1994. Vaccination of sheep against Fasciola hepatica with glutathione s-transferase. J. Immunol. 152: 1861-1872.

139. Moore J, Sattentau Q, Wyatt R and Sodroski J. 1993. Probing the structure of HIV surface glycoprotein gp120 with a panel of monoclonal antibodies. J. Virol. 68: 469-484.

140. Moore JP, Yoshiyama H, Ho DD, Robinson JE and Sodroski J. 1993. Antigenic variation in gp120s from molecular clones of HIV-1 LAI. AIDS Res. Hum. Retroviruses. 9: 1185-1193.

141. Earl PL, Broder CC, Long D, Lee SA, Peterson J, Chakrabarti S, Doms RW and Moss B. 1994. Native oligomeric human immunodeficiency virus type 1 envelope glycoprotein elicits diverse monoclonal antibody reactivities. J. Virol. 68(5): 3015-3026.

142. Geysen HM, Mason TJ and Rodda S. 1988. Cognitive features of continuous antigenic determinants. J. Molec. Recog. 1(1): 32-41.

143. Naray-Szabo G. 1993. Analysis of molecular recognition: steric electrostatic and hydrophobic complementarity. J. Molec. Recog. 6: 205-210.

144. Churchill ME, Stura EA, Pinilla C, Appel JR, Houghten RA, Kono DH, Balderas RS, Fieser GG, Schulze-Gahmen U and Wilson IA. 1994. Crystal structure of a peptide complex of anti-influenza peptide antibody Fab 26/9. Comparison of two different antibodies bound to the same peptide antigen. J. Mol. Biol. 241: 534-556.

145. Pestano G, Spira A, Hosford K, Riley J, Prince A, Brown L and Boto, W. 1994. Antigenic peptide distribution in the gp120 sequences of divergent African and North American HIV-1 subtypes. Keystone Symposia on Molecular and Cellular Biology. Hilton Head Is., SC. J. Cell. Biochem. (Supplement 18B), Abstract No. J118.

146. Vogel T, Kurth R and Norley S. 1994. The majority of neutralizing Abs in HIV-1 infected patients recognize linear V3 loop sequences. J. Immuol. 1895-1904.

147. Hosford K, Pestano G, Lee S-K, and Boto W. 1995. Envelope gp120 molecules in HIV-1 subtypes A through D encode analogous antigenic epitopes. In Third Annual Smith Kline Beecham/Student National Medical Association (SNMA) Region VII Research Forum Conference. University of Massachusetts Medical School, Worchester, MA.

148. Pestano G, Hosford K, Lee S-K, Samms M, Usavage K, Campbell K, Riley J, Guyden J and Boto WMO. 1996. Molecular analyses of the V3 PND sequences in HIV-1 clades A through D. In Keystone Symposia on HIV Pathogenesis. Hilton Head Is. SC. Abstract No. E 360.

149. White-Scharf ME, Potts BJ, Smith LM, Sokolowski KA, Rusche JR and Silver S. 1993. Broadly neutralizing monoclonal antibodies to the V3 region of HIV-1 can be elicited by peptide immunization. Virology. 192: 197-208.

150. Wilmot CM and Thornton JM. 1988. Analysis and prediction of the different types of β-turns in proteins. J. Mol. Biol. 203: 221-232.

151. Mascola JR, Louwagie J, McCutchan FE, Fischer CL, Hegerich PA, Wagner KF, Fowler KF, McNeil JG and Burke DS. 1994. Two antigenically distinct subtypes of HIV-1: viral genotype predicts neutralization serotype. J. Infect. Dis. 169: 48-54.

152. Golding H, D'Souza MP, Bradac J, Matthieson B, and Fast, P. 1994. Neutralization of HIV-1. AIDS Res. Hum. Retroviruses. 10: 633-643.

153. Delwart EL, Shpaer EG, Louwagie J, et. al. 1993. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 env genes. Science. 262: 1257-1261.

154. Bachmann MH, Delwart EL, Shpaer EG, Lingenfelter P, Singal R, Mullins JI and the WHO Network for HIV Isolation and Characterization. 1994. Rapid genetic characterization of HIV type 1 strains from four World Health Organization-sponsored vaccine evaluation sites using a heteroduplex mobility assay. AIDS Res. Hum. Retroviruses. 10(11): 1345-1353.

155. Garnier J. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of glubular proteins. J. Mol. Biol. 120: 97-120.

156. Sternberg MJE and Zvelbil MJ. 1990. Prediction of protein structure from sequence. Eur. J. Cancer. 26(11/12): 1163-1166.

157. Hopp TP. 1994. Retrospective: 12 years of antigenic determinant predictions, and more. Peptide Res. 6: 183-190.

158. Van Regenmortel HMV and Pellequer J-L. 1994. Predicting antigenic determinants in proteins: looking unidimensional solutions to a three-dimensional problem? Peptide Res. 7: 224-228.

159. Benner SA. 1995. Predicting the conformation of proteins from sequences. Progress and future progress. J. Molec. Recog. 8: 9-28.

160. Atassi MZ. 1978. Precise prediction of the entire antigenic structure of lysozyme. Molecular features of protein antigenic structures and potential of "surface stimulation" synthesis-a powerful new concept for protein binding sites. Immunochemistry. 15: 909-936.

161. Harper DO. 1995. Molecular Virology. Bios Press, UK.

162. Jansson M, Wahren B, Albert J, Scarlatti G, Frazen L, Kathaa PK, Katabira E, Mubiro F, Wigzell H and Rossi P. 1994. Peptide serology for analysis of the inter-and intra-individual variation in the HIV-1 V3 domain. AIDS. 8: 413-421.

163. Smith JD, Bruce ASR, Featherstone RG, Downing RG, Biryahawho B, Clegg JCS, Carswell JW and Oram JD. 1994. Reactions of Ugandan antisera with peptides encoded by V3 loop epitopes of human immunodeficiency virus type 1. AIDS Res. Hum. Retroviruses. 10: 577-583.

164. Carrow EW, Vujcic LK, Glass WL, Seamon KB, Rastogi SC, Hendry RM, Boulos R, Nzila N and Quinnan GV. 1991. High prevalence of antibodies to the gp120 V3 region principal neutralizing determinant of HIV-1MN in sera from Africa and the Americas. AIDS Res. Hum. Retroviruses. 7(10): 831-838.

165. Baillou A, Brand D, Denis F, M'Boup S, Chout R, Goudeau A and Barin F. 1993. High antigenic cross-reactivity of the V3 consensus sequences of HIV-1 gp120. AIDS Res. Hum. Retroviruses. 9: 1209-1215.

166. Cheingsong-Popov R, Lister S, Callow D, Kaleebu P, Beddows S, Weber and the WHO network for HIV isolation and characterization. 1994. Serotyping HIV type 1 by antibody binding to the V3 loop: relationship to viral genotype. AIDS Res. Hum. Retroviruses. 10: 1379-1386.

167. Pau C-P, Kai M, Holloman-Candal D, Luo C-C, Kalish ML, Schochetman G, Byers B, George JR and the WHO network for HIV isolation and characterization. 1994. Antigenic variation and serotyping of HIV type 1 from four World Health Organization-sponsored HIV vaccine sites. AIDS Res. Hum. Retroviruses. 10: 1369-1377.

168. Korber BTM, McInnes K, Smith RF and Myers G. 1994. Mutational trends in the V3 loop protein sequnces observed in different genetic lineages of human immunodeficiency virus type 1. J. Virol. 68(10): 6730-6744.

169. Pestano GA, Hosford K, Riley J, Spira A, Mascola J, Guyden J, Ho D, and Boto WMO. 1995. Conservation of antigenic specificities in divergent V3 loop residues from HIV-1 clades A through F. In Keystone Symposia on HIV Pathogenesis. Keystone, CO. J. Cell. Biochem. (Suppl. 21B). Abstract No. D4-336.

170. Gupta G, Anantharamaiah GM, Scott DR, Eldridge JH and Myers G. 1993. Solution structure of the V3 loop of a Thailand HIV isolate. J. Biomol. Struct. Dyn. 11: 345-66.

171. Berger EA, Sisler JR and Earl PL. 1992. Human Immunodeficiency Virus type 1 envelope glycoprotein molecules containing membrane fusion-impairing mutations in the V3 region efficiently undergo soluble CD4-stimulated gp120 release. J. Virol. 66(1): 6208-6212.

172. Page K, Stearns SM and Littman DR. 1992. Analysis of mutations in the V3 domain that affect fusion and infectivity. J. Virol. 66(1): 524-533.

173. Ebenbichler C, Westerveldt P, Carillo A, Henkel T, Johnson D and Ratner L. 1993. Structure-function relationships of the HIV-1 envelope V3 loop tropism determinant: evidence for two distinct conformations. AIDS. 7: 639-646.

174. Stamatatos L and Cheng-Mayer C. 1993. Evidence that the structural conformation of the envelope gp120 affects human immunodeficiency virus type 1 infectivity, host range and synctium-forming ability. J. Virol. 67(9): 5635-5639.

175. Clements GJ, Price-Jones MJ, Stephens PE, Sutton C, Schultz TF, Clapham PR, McKeating JA, McClure MO, Thompson S, Marsh M, Weiss RA and Moore JP. 1991. The V3 loops of HIV-1 and HIV-2 surface glycoproteins contain proteolytic cleavage sites: A possible function in viral fusion? AIDS Res. Hum. Retroviruses. 7: 3-16.

176. Pal R, diMarzo V, Nair BC, Rahman R, Hoke G, Mumbauer SW and Sarngadharan MG. 1992. Characterization of a neutralizing monoclonal antibody to the external glycoprotein of HIV-1. Intervirology. 34: 86-93.

177. Lee S-K. 1996. Characterization of immunogenic properties of divergent HIV-1 subtypes. Master of Arts Thesis. Biology Department, The City College of The City University of New York, NY.

178. Samms M, Pestano G, Lee S-K, Riley J and Boto WMO. 1995. Comparison of MAP and DNA plasmid immunization for HIV-1 vaccine development. In Annual NIGMS Scholars Meeting, Washington, D.C. Abstract No. D64.

179. VanCleave VH, Naeve CW and Metzger DW. 1984. Do antibodies recognize amino acid side chains of protein antigens independently of the carbon backbone? PNAS (USA). 81: 3998-4002.

180. Geysen HM, Mason TJ and Rodda SJ. 1988. Cognitive features of continuous antigenic determinants. J. Molec. Recog. 1(1): 32-41.

181. Page M, Vella C, Corcoran T, Dilger P, Ling C, Heath A and Thorpe R. 1992. Restriction of serum antibody reactivity to the V3 neutralizing domain of HIV gp120 with progression to AIDS. AIDS. 6: 441-446.

182. Geyer H, Holschbach C, Hunsmann G and Schneider J. 1988. Carbohydrates of human immunodeficiency virus. J. Biol. Chem. 263: 11760-11767.

183. Bernstein HB, Tucker SP, Hunter E, Schutzbach JS and Compans RW. 1994. Human immunodeficiency virus type 1 envelope gylcoprotein is modified by O-linked oligosaccharides. J. Virol. 68(1): 463-468.

184. Bach NKT, Smit L, Dejong JJ, et al. 1994. An N-glycan within the human immunodeficiency virus type 1 gp120 V3 loop affects virus neutralization. Virology. 2: 431-438.

185. Alexander S and Elder H. 1984. Carbohydrate dramatically influences immune reactivity to antisera of viral glycoprotein antigens. Science. 226: 1328-1330.

186. Weiss CD, Levy JA and White JM. 1990. Oligomeric organization of gp120 on infectious human immunodeficiency virus type 1 particles. J. Virol. 64(1): 5674-5677.