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Immunogenic potential of rgp120 from African HIV-1 subtype A

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Previous studies have shown that the African strains of HIV-1 mostly cluster with the subtypes A, C or D based on phylogenetic analysis of the ENV nucleotide sequences. In the present investigation we have examined the immunogenic potential of full length gp120 derived from the Ugandan HIV-1 subtype A isolate, AUG06c, using computer-based prediction methods and a plasmid-mediated immunization technique. Computer-assisted analysis of the amino acid residues identified 15 potential B-cell epitopes in gp120 of AUG06c. Despite marked variation in the primary sequences, these epitopes were shown to correspond well to analogous sites in gp120 derived from the subtype B reference clones, MN and $IIIB_{BH10}$. The relative positions of the epitopes indicated that E9[V3], E14[C3] and E15[V5] correspond to the previously defined principal neutralizing determinant (PND) located in the V3 loop, the CD4 binding site and gp120 "immunodominant" region, respectively. Intramuscular inoculation of BALB/c mice with the ENV clones from AUG06c or from the subtype C clone, CUG045 elicited antibodies which react with the homologous but not with the heterologous PND peptide in ELISA. However, cocktail inoculation with the ENV plasmids from AUG06c and CUG045 elicited antibodies which reacted with both peptides. Antibody response to the other predicted epitopes of AUG06c was not as strong as the response to the PND peptide. The response of the mice to DNA-mediated immunization was further tested in a proliferation assay. Spleen cells derived from the immunized mice exhibited a strong proliferative response to homologous and heterologous PND peptides in [^{3}H]thymidine incorporation assay. DNA-mediated immunization with rgp120 of AUG06c appears to elicit cellular immune response of relatively broad specificity. Copyright © 1996 Elsevier Science Ltd.

Keywords: HIV; DNA vaccine; rgp120

Brazil, Rwanda, Thailand and Uganda have been recently proposed as the study sites for the clinical trial of the next generation of HIV-1 vaccines[1]. Two major obstacles have been identified in the current search for immunoprophylactic agents to control the escalation of the AIDS epidemic. One of these concerns is the presumed impact of strain variation on the neutralizing efficacy of the leading candidate immunogen, gp120; the other is the emerging need to develop a safe vaccine formulation that would assure long-term *in vivo* availability of immunogenic peptides.

Nucleotide sequence analysis of the ENV gene has revealed worldwide distribution of multiple divergent HIV-1 subtypes[1–4]. These reports[1,3,4] together with the ENV sequences available in the Human Retrovirus and AIDS Database[2] indicate that the genetic clades A and D constitute the largest proportion of the isolates from Uganda, Rwanda and Zaire. The Brazilian clones analyzed to date are predominantly of the B and F subtypes, whereas the Thai strains are of the B and E subtypes[2]. Isolates comprising the B clade are by far the most common in South and North America, the Caribbean and western Europe[2]. Relatively minor clades also contribute to the infection in the African localities. The subtype C virus has been described recently in Uganda, Zambia and southern African regions[2,5]. Strains from the clades B, E, F, G, H and the outgroup O exist in many regions of Central and West Africa[2,4,6]. The D and O subtypes apparently exhibit the most extensive intra-clade sequence diversity[1–6]. The presence of all of the major subtypes of HIV-1 in the African locales has greatly compounded the current search for efficacious candidate immunogens.

The isolation and characterization of broadly reactive gp120 molecules could complement the current international search for candidate vaccines that would be suitable for worldwide application. Studies previously reported from this laboratory have shown that the subtype A isolate, AUG06c, reacts with a significant proportion of the sera from asymptomatic Ugandan

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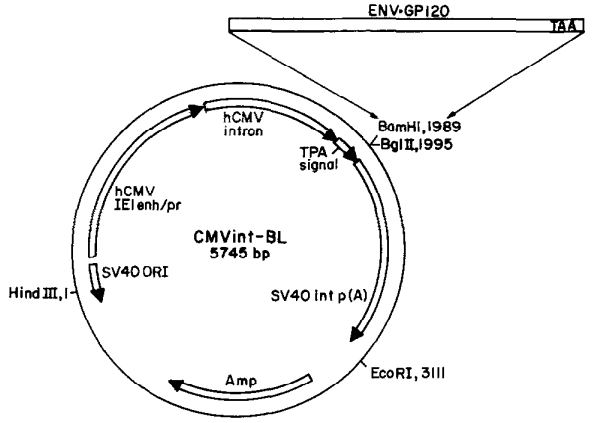

Figure 1 Map of CMVint-BL showing the ligation site for the ENV.GP120 fragments. The clone was ligated at the BamH1 site immediately following the TPA sequence. Expression was driven from the start codon of TPA. The stop codon (TAA) in gp120 is shown

donors[7,8]. In the present investigation we have cloned the full-length gp120 from AUG06c into a CMV promoter-driven expression vector. The findings presented in this report indicate that this clone of gp120 expresses multiple antigenic sites analogous to those found in the reference subtype B clones, MN and $IIIB_{BH10}$ and elicits both humoral and cellular immune responses.

MATERIALS AND METHODS

Molecular cloning of ENV.GP120

Full-length ENV.GP120 was cloned from the subtype A isolate, AUG06c, which has been well characterized in previous studies[5,7,8]. The antigenic epitope(s) encoded in AUG06c appears to be well conserved among some of the Ugandan strains as shown by the level of cross reactivity with sera from indigenous donors in ELISA[5] and in neutralization assay[7,8]. AUG06c was expanded in culture by infection of PHA-stimulated normal peripheral blood leukocytes[7,8]. Genomic DNA was isolated from the lysates of infected cells by phenol–chloroform extraction and then used to construct a lambda phage recombinant library[9]. The plaques were screened with ^{32}P-labeled ENV DNA from $IIIB_{BH10}$. Nested primers were used to amplify the full length ENV.GP120 (1.6 kb) from a positive plaque. Both the 5′ and 3′ primers were synthesized to include BamH1 restriction site. PCR was performed as previously described[5,7,8]: denature (95°C) 30 s; anneal (50°C) 30 s; extend (72°C) 1 min for 35 cycles; and a final extension at 72°C for 10 min. The PCR product was gel-purified and then cloned into an expression vector for immunization.

Construction of expression vector

The plasmid, CMVint-BL, was previously constructed by Chapman *et al.*[10] and subsequently modified to include several additional cloning sites[11] (*Figure 1*). The plasmid contains the SV40 origin of replication, human cytomegalovirus (Towne strain) major immediate–early gene enhancer/promoter (hCMV IE1) and intron A[10]. The hCMV IE1 promoter is among the strongest viral gene regulators available for transient expression of diverse heterologous proteins[10,12–18]. The versatility in the performance of hCMV IE1 has been attributed to the functions of a number of motifs in the DNA[10,12–18]. Sequences upstream of the promoter include multiple potential binding sites for many well-known eukaryotic transcriptional control factors such as nuclear factor1 (NF1). These factors are present in cells of diverse tissue origins including muscles[10,12–18]. Further, intron A contains a binding site for NF1. Thus both intron A and the human tissue plasminogen activator (TPA) signal sequence in CMVint-BL have been shown to contribute to increased secretion of several heterologous reporter glycoproteins, including rgp120 of HIV-1[10]. The 5′ primer used for PCR in this study was designed to exclude the gp120 signal peptide sequence and transcription start codon. The function of the gp120 signal peptide is apparently inhibited in the absence of rev[10].

The PCR-amplified ENV.GP120 was ligated at BamH1 site upstream of the SV40 small t polyadenylation sequence (*Figure 1*)[10] according to standard restriction digestion and cloning procedures[9]. Similarly, truncated fragments of ENV.GP120 derived from the divergent clones CUG045 (650 bp, subtype C)[5] and DUG23c (725 bp, subtype D)[7,8] were amplified from the pT7Blue vector[5,7,8] and inserted into CMVint-BL at the BamH1 site. It was previously reported that these fragments encode the C2 to the V5 domains[5,7,8]. The region overlaps the major immunogenic sites found in gp120[5,7,8]: the PND located in the V3 loop, the neutralizing CD4 binding site, gp120 "immunodominant" region and at least six other B-cell epitopes[5]. A cytotoxic T-cell epitope has been mapped to a region within the V3 loop[19]. The in-frame ligation of ENV.GP120 and the truncated clones was confirmed by DNA sequencing. The resulting recombinant plasmids from clones AUG06c, CUG045 and DUG23c were designated as CMV/AUG06c, CMV/CUG045 and CMV/DUG23c, respectively. The sequences for AUG06c, DUG23c and CUG045 are available in GenBank under the accession numbers M98504, M98503 and U11597, respectively.

Transient expression of rgp120 peptides

Freshly passaged CEM (CD4) cells were transfected with the respective ENV plasmid using a liposome-mediated protocol (Boeringher Mannheim, IN). The cells (3×10^5 ml^{-1}) were pelleted by centrifugation for 10 min at 250*g*. The supernatant was removed prior to the addition of the transfection mixture to the cell pellet. 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. The entire solution (150 μl) was added to 5 ml of RPMI 1640 culture medium (JRH Biosciences, KS) supplemented with 10% fetal bovine serum, 2% penicillin/streptomycin (JRH Biosciences), 0.1 mM nonessential amino acids and 2 mM L-glutamine (GibcoBRL, NY). The cultures were incubated for a maximum of 72 h at 37°C in 5% CO_2 and 95% humidity. Cells were harvested at 24 h intervals, lysed, and the protein extracts were quantified by O.D. measurement at 600 nm using the modifications of the Bradford method[20] as described in the Quantify Protein Assay System (Promega, WI). The expression of the rgp120 peptides was monitored using ELISA which was

conducted as previously described[21]. One hundred microliters of the extract (50 μg ml^{-1}) diluted in 0.1 M $NaHCO_3$ (pH 9.6) were used to sensitize 96-well polyvinyl microtiter plates overnight at 4°C. Antibody reactivity of the cell extract was determined using previously characterized hyperimmune serum samples from HIV-1 infected Ugandan donors[5]. Peroxidase labeled rabbit anti-human whole IgG was used as the indicator. Background values were obtained by reacting normal human sera simultaneously with the test samples. The negative control values (<10% of test sample) were subtracted from the experimental values to facilitate sample to sample comparison.

DNA inoculation

BALB/c mice were inoculated in the quadriceps muscle[22] and then boosted at 2-week intervals with doses of 100 μg of each plasmid in 200 μl of isotonic saline. A 1 cm^3, 28G insulin syringe (Beckton Dickinson, NJ) was used to administer the plasmid DNA. Groups of four mice were inoculated with single clones or a cocktail of CMV/AUG06c, CMV/CUG045 and CMV/DUG23c. The cocktail contained 100 μg of each plasmid in a total volume of 200 μl of saline. Control mice were inoculated with CMVint-BL without the ENV insert. The animals were cared for in accordance with institutional guidelines. Blood (300–500 μl) was sampled from the tail vein at 4-week intervals. The blood samples were allowed to clot at 4°C overnight and then centrifuged at 10000 revs min^{-1} for 20 min. Serum samples were collected and frozen at −20°C until needed.

Prediction of linear antigenic epitopes in full length gp120

Amino acid translations were conducted using PC/Gene and positions were numbered according to the sequence in AUG06c. Linear antigenic sites were identified using the algorithms in SURFACE PLOT (version 1.4) and POLAR TRIPEPTIDE in PPSP (Synthetic Peptides Inc., Canada). SURFACE PLOT predicts surface and interior sites for tripeptide residues in a protein backbone based on a composite value for hydrophilicity, flexibility and accessibility[23]. POLAR TRIPEPTIDE determines high probability surface residues utilizing values for surface exposure obtained from the Brookhaven Database of protein X-ray crystallographic structures. High (60%) and intermediate (25%) probability cutoffs were used to generate the antigenic residues reported herein. Regions predicted to contain three or more contiguous "hidden" residues were not considered to be antigenic in these analyses. The Chou–Fasman algorithm in the BETATURN program[24] was used to obtain the antigenic probability of the V3 loop residues.

Synthesis of peptides

Linear peptides comprising the predicted PND in gp120 were synthesized on an automated peptide synthesizer (Synergy, Model 432A, ABI) using the solid-phase procedure[25] as previously reported[5]. Peptides from the following clones were synthesized: AUG06c (RPYKKVRRRKHIGPGRSFY), CUG045 (RPNNNTRESVRIGPGQAFY), DUG23c (RPYENVRHRTPIGLGQALI) and MN (CNKRKRIHIGPGRAFYTTKN) (Intracel, MA). In all instances, the fidelity of synthesis was monitored by analyzing the conductivity traces generated during peptide synthesis cycles. Post-synthesis cleavage of the peptide from the resin and protecting groups was conducted using an aqueous solution containing thioanisole, trifluoroacetic acid and dithioethane as scavengers. The peptides were filtered through glasswool, precipitated in cold methyl–terbutyl ether (MTBE, Sigma, MO) and extracted in aqueous acetic acid. Three more washes of the peptide with MTBE were performed to remove any traces of contaminants. Further purification of the products has not been found necessary for optimal reactivity in ELISA.

ELISA for mouse antibodies

Duplicate assays were conducted using the predicted PND and other linear peptides in gp120. The assay was performed essentially as described[26]. Horseradish peroxidase-labeled goat anti-mouse whole IgG (Promega, WI) was used as the indicator reagent. O.D. readings were determined at 492 nm. Values from duplicate assays varied by <10%. Control mice were inoculated with CMVint-BL without the ENV insert.

Proliferation assay

Spleens were aseptically removed from the mice and teased in sterile medium[27]. The cells were washed twice with RPMI 1640 culture medium supplemented with 2 mM glutamine and 20 mM HEPES. The splenocytes were then adjusted to 3×10^6 cells ml^{-1} in complete T cell medium [equal volumes of RPMI 1640 and EHAA (GibcoBRL, NY), 10% fetal bovine serum, 5×10^{-5} M β-mercaptoethanol (Sigma), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin]. Two hundred microliters of the cell suspension were cultured in triplicate in 96-well microculture plates with the respective peptides (30 μg ml^{-1}) or PHA (5 μg ml^{-1}) or mouse rIL2 (20 U ml^{-1}, Sigma), for 3 days at 37°C in 5% CO_2 and 95% humidity. The cells were then pulsed by the addition of 1 μCi of [^{3}H]thymidine (DuPont, DE). Radiolabeled DNA was harvested on glass fiber filter paper after 20 h of pulse. Samples were air-dried and the isotope was quantified using a liquid scintillation counter. [^{3}H]Thymidine incorporation was expressed in counts per minute (c.p.m.).

RESULTS

Multiple B-cell epitopes expressed in full-length gp120 of AUG06C

The potential effects of sequence variation on the distribution and composition of the predicted B-cell epitopes in gp120 of AUG06c and the reference clones were examined (*Figure 2*). The full-length gp120 molecule (520 a.a.) encoded in AUG06c exceeds those of the reference clones $IIIB_{BH10}$ and MN. Fifteen antigenic sites (E1-E3, E5-E16) were discerned in gp120 of AUG06c. Despite the apparent divergence in the primary sequences, AUG06c, MN and BH10 were found to express analogous epitopes in terms of their

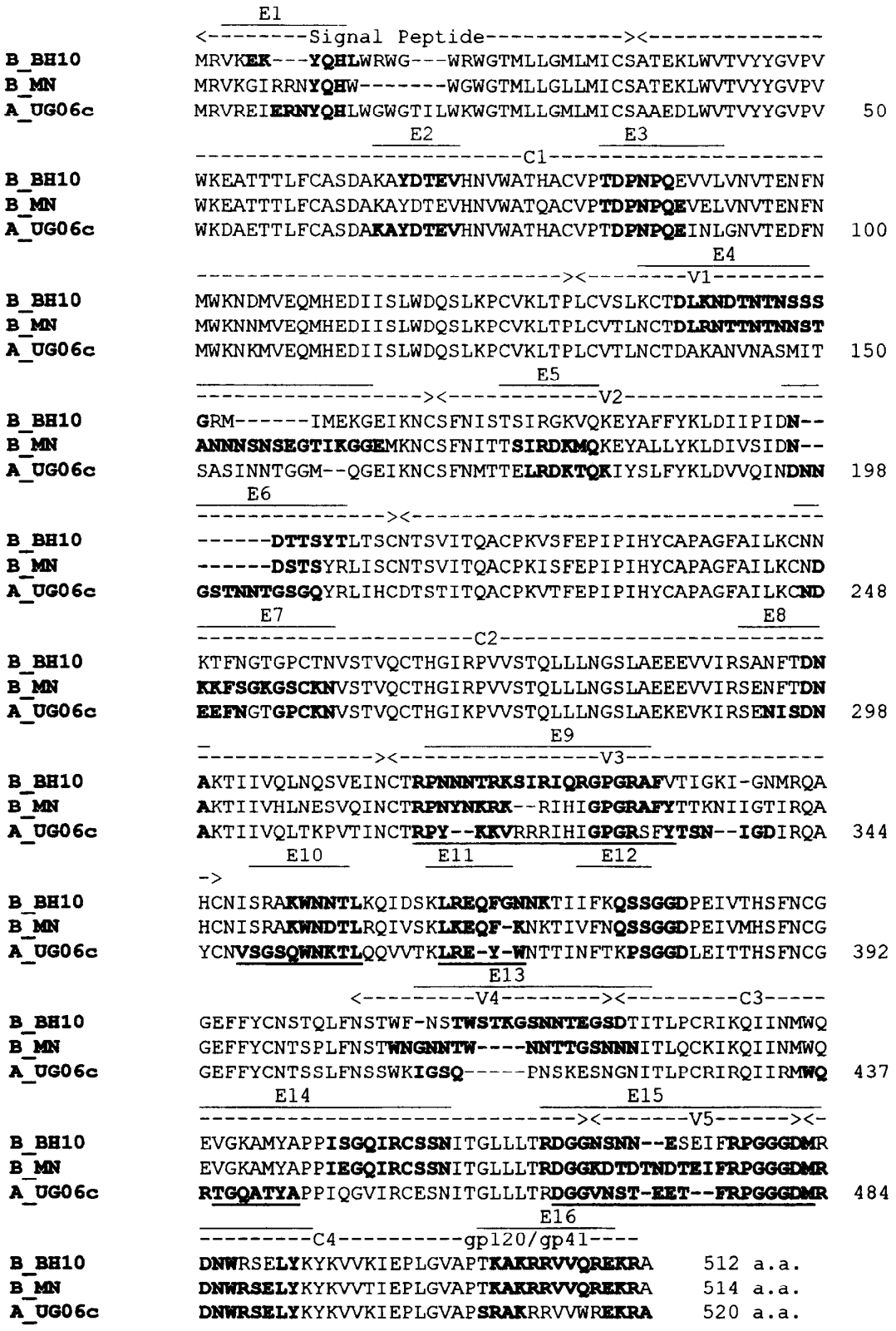

Figure 2 The predicted linear antigenic epitopes E1 through E16 are shown for full lengh gp120 from AUG06c and for the subtype B reference clones, MN and BH10. Residues of high antigenic potential were determined using the complementary programs SURFACE PLOT, POLAR TRIPEPTIDE and BETATURN (Materials and Methods). Symbols: -, gap introduced to facilitate sequence alignment; underlined sequences were synthesized for use in ELISA and proliferation assay. Domain assignment is according to Modrow *et al.*[30]

relative locations on gp120. However, the reference clones MN and BH10 express an additional epitope, E4[V1], which is lacking in AUG06c.

The variations in the length and in the primary sequences were clearly attributable to multiple deletions and substitutions observed throughout the molecules of

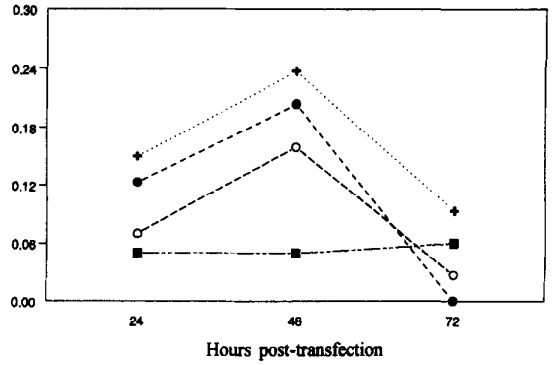

Figure 3 ELISA reactivity of rgp120 peptides expressed in transfected CEM cells. Transfections were conducted with the following plasmids: CMV/AUG06c (○); CMV/CUG045 (●); and CMV/DUG23c (+). Control cells were transfected with CMVint-BL without ENV (■). Background reactivity of normal human serum antibodies with the cell extracts was subtracted from experimental values

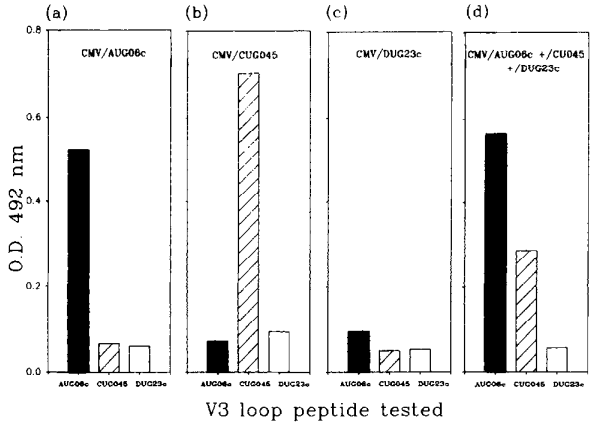

Figure 4 ELISA reactivity of the predicted PND synthetic peptide from AUG06c, CUG045, or DUG23c with the individual sera from mice inoculated with the ENV plasmids. Representative reactivities of serum samples from groups of four mice inoculated with: CMV/AUG06c only (a); CMV/CUG045 only (b); CMV/DUG23c only (c) or CMV/AUG06c+CMV/CUG045+CMV/DUG23c in a cocktail (d). The serum samples were tested with V3 peptides encoded in AUG06c (□), CUG045 (hatched column), and DUG23c (■). At least 50% of the mice in each group seroconverted by 1 month post priming. The pattern of reactivity of serum from the other mice was not different (data not shown). O.D. readings for the control sera (<5% of test values) were subtracted from all test samples

either clone. The sequences comprising the epitopes E1 in the signal peptide, E2[C1], E3[C1], E8[C2] and E14[C3] are relatively conserved; the other sites display moderate to marked sequence diversity. The relative positions of the predicted antigenic sites indicate that E9[V3], E14[C3] and E15[V5] comprise the previously determined V3 loop PND, residues in the CD4 binding site and gp120 "immunodominant" region, respectively[5]. The other epitopes presented here (*Figure 2*) have not been characterized in previous studies.

In vitro expression of rgp120 by plasmid constructs

The plasmid constructs CMV/AUG06c, CMV/CUG045 and CMV/DUG23c were transfected into the T-cell line, CEM. Lysates of the cells were tested in ELISA for the presence of rgp120 using hyperimmune human sera (*Figure 3*). Serum antibodies detected rgp120 in the lysates of cells transfected with each plasmid. Extracts from cells that were transfected with a plasmid lacking ENV.GP120 or truncated clones did not react with these sera. Maximal reactivity was observed by 48 h (*Figure 3*). The eukaryotic expression of gp120 has been previously reported to be rev-dependent[19,28]. Our findings have provided additional evidence for a rev-independent expression of rgp120 by CMVint-BL[10]. The vector has been previously shown to boost the expression and secretion of the glycoprotein many-fold higher than the levels achieved using the SV40 early enhancer/promoter system[10].

Antibody response to plasmid-mediated immunization with rgp120

To assess the immunogenic potential of rgp120, the plasmids CMV/AUG06c, CMV/CUG045 and CMV/DUG23c, were inoculated into the quadriceps muscle of BALB/c mice. Inoculation with CMV/AUG06c alone elicited antibodies which react with the homologous V3 PND peptide but not with the divergent peptide from CUG045 or DUG23c (*Figure 4*a). Likewise, inoculation with CMV/CUG045 alone elicited antibodies which recognize the homologous PND peptide, but hardly react with the analogous peptides from AUG06c or DUG23c (*Figure 4b*). In contrast, inoculation with CMV/AUG06c, CMV/DUG045 and CMV/DUG23c in a cocktail induced antibodies that react with V3 peptide in AUG06c and CUG045 (*Figure 4d*). Antibodies induced by the combination immunogen recognized the PND peptides from both AUG06c and CUG045, but failed to react with the analogous peptide from DUG23c (*Figure 4d*). Inoculation with CMV/DUG23c generated near-background levels of antibody response in these studies (*Figure 4c*). These patterns of antibody response and reactivity to single or cocktail immunization have been confirmed in several other experiments (not shown). DNA-mediated immunization elicited V3 loop antibodies of variable titers. Representative ranges of values from single or cocktail inoculation are shown when tested against the homologous V3 loop PND peptide from AUG06C (*Figure 5*). Reciprocal antibody titers of 10000–20000 have been observed following cocktail immunization. Inoculation with the plasmid cocktail generally provoked a more vigorous and rapid antibody development.

Immunogenic potential of the predicted antigenic epitopes in gp120

Multiple linear epitopes have been previously identified in gp120 based on several prediction methods[5]. The relative immunogenic potential of these putative antigenic sites was examined. Synthetic peptides comprising the epitope designated E9[V3] indicated a strong antibody reactivity (*Figure 6*). The PND has been previously mapped to the E9[V3] site in gp120[5]. Similar peptides derived from all other epitopes also elicited an antibody response, though with less intensity compared to E9[V3]. The patterns of reactivity of these epitopes with the mouse antibodies closely parallel those of hyperimmune human sera[5]. DNA-mediated immunization apparently presents the same antigenic sites of gp120 recognized by humans in a natural infection.

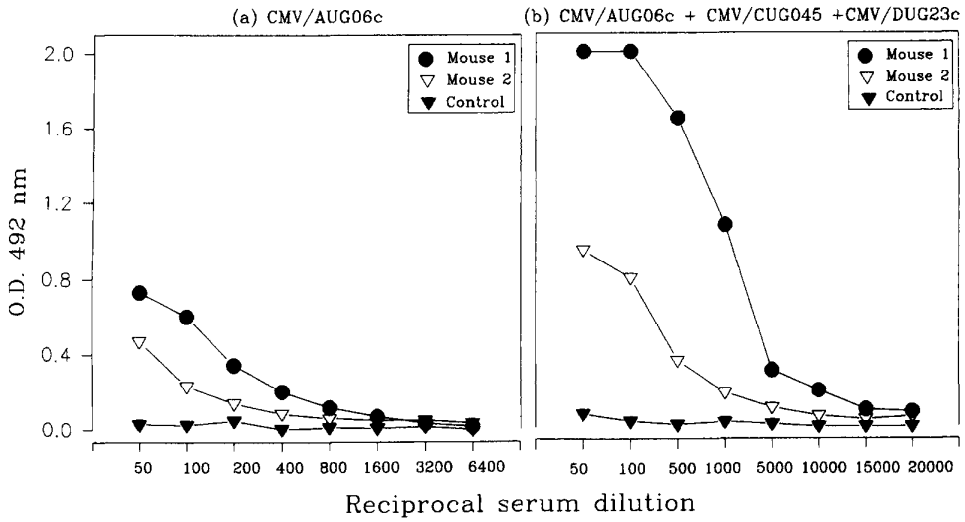

Figure 5 Reactivity of the V3 PND peptide from AUG06c with sera from mice inoculated with CMV/AUG06c only (a) or CMV/AUG06c+CMV/CUG045+CMV/DUG23c in a cocktail (b). Mice were boosted at 2-week intervals and then bled at 3 months post initial inoculation for the experiment

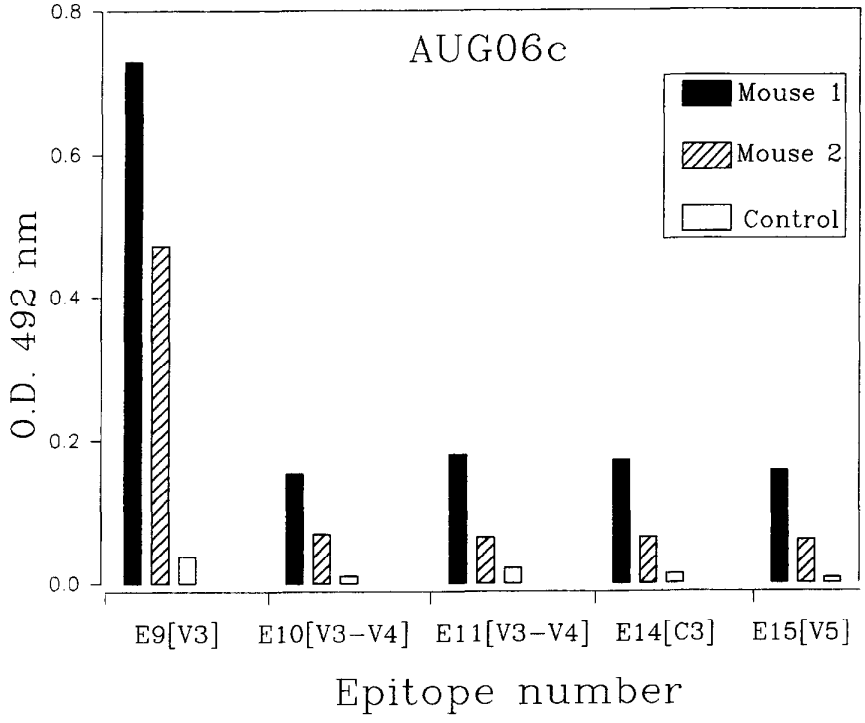

Figure 6 ELISA reactivities of mouse antibodies with the predicted epitopes of gp120 from AUG06c. Serum samples were tested against synthetic peptides comprising the epitopes E9[V3] (residues 316–334), E10[V3–V4] (348–357), E11[V3–V4] (364–368), E14[C3] (439–445) and E15[V5] (466–483). GP120 domains are indicated in brackets. Each bar represents the mean value of duplicate assays. Duplicate assays varied by <5% in this test

Induction of proliferative response by CMV/AUG06C

Spleen cells were recovered from the mice previously inoculated with CMV/AUG06c and then incubated with the PND peptide from AUG06c or CUG045 or MN. When cultured without a stimulus in the medium, spleen cells from the inoculated mice incorporated more [^{3}H]thymidine than cells from nonimmunized mice (*Table 1*). The spontaneous proliferation of the splenocytes from the immune mice could be attributed to the presence of antigen-primed lymphocytes undergoing division at the point of isolation. If this interpretation is correct, then such cells would be expected to proliferate further when supplied with exogenous rIL2 alone as shown (*Table 1*). The potency of the peptide from AUG06c to promote the proliferation of the immune spleen lymphocytes was evident (*Table 1*). The response to the PND peptide of the divergent subtypes CUGO45 and MN was similarly significant. rIL2 synergized with the peptides in every case to enhance [^{3}H]thymidine incorporation. The results suggest that IL2 is a potential limiting factor in the response of spleen cells to gp120, at

Table 1 Incorporation of [^{3}H]thymidine in spleen cells of mice inoculated with the plasmid CMV/AUG06c

Stimulus	[^{3}H]Thymidine incorporation (c.p.m.)		
	Immunized mouse 1	Immunized mouse 2	Non-immunized
None (medium only)	973±108	1680±238	126±31.6
rIL2	2086±408	N.A.[a]	140±4
AUG06c	2424±27	9596±2281	396±188
AUG06c+rIL2	3052±273	N.A.[a]	318±35
CUG045	1556±916	9786±1953	105±10
CUG045+rIL2	3578±1512	N.A.[a]	316±71
MN	1460±327	N.A.[a]	116±15
MN+rIL2	3968±104	N.A.[a]	209±29
PHA	21958±1135	43512±2975	N.A.[a]

The cells were stimulated with homologous or heterologous PND peptide at 30 μg ml^{-1}, or PHA (5 μg ml^{-1}); with or without rIL2 (20 U ml^{-1}). Each value represents the mean and standard deviation of triplicate assays. [a]Not available

least *in vitro*. Thus, the response to the synthetic peptide antigens was much less impressive when compared to the mitogenic activity of PHA (*Table 1*).

DISCUSSION

The complementary algorithms for surface probability (flexibility, hydrophilicity, accessibility and β-turn conformation) were used to predict potential B-cell epitopes in full length gp120 encoded in AUG06c. These parameters have been previously shown to predict antigenic sites in various proteins[23,24] including gag[29] and gp120[5,30] of HIV-1. Analogous antigenic sites were identified in gp120 of AUG06c, MN and BH10 (*Figure 2*) by these methods. The relative positions of the epitopes were shown to correspond well to those previously defined for the laboratory-adapted HIV-1 isolates using similar prediction methods[30]. Many of the epitopes described in this report were shown to exhibit marked sequence variability, while others were relatively conserved in different virus subtypes. As expected, the epitope E9[V3] corresponding to the PND was among the more variable positions (*Figure 2*).

An important goal of the current search for immunoprophylactic agents to control the dissemination of HIV-1 is the development of a safe vaccine formulation that would assure long-term *in vivo* release of immunogenic peptides. In the present investigation we have assessed the immunizing potential of rgp120 from AUG06c using a plasmid-mediated immunization technique. Both antibody and lymphocyte proliferative responses to the homologous PND and many other peptides were recorded. Our findings raise the possibility that this format of immunization with rgp120 could elicit both humoral and cellular immune responses in other animals including humans. These results also support previous studies indicating that plasmid-mediated immunization with the ENV clones from the laboratory-adapted isolate, IIIB, elicits ELISA-reactive antibodies and CTL reactivity in mice[31,32]. However, this is the first report to show that a cocktail inoculation with ENV.GP120 plasmids from well-characterized field isolates[5,7,8] can also provoke similar responses.

We have previously investigated the antigenic properties of gp120 encoded in AUG06c, CUG045 and DUG23c using hyperimmune human sera in ELISA[5] and in neutralization assay[7,8]. These earlier studies had shown that the epitopes presented in AUG06c, CUG045 and DUG23c are quite antigenic and appear to be relatively conserved in at least some of the Ugandan strains[5]. In the present study, however, inoculation with only CMV/AUG06c or CMV/CUG045 was shown to induce mostly type-specific anti-V3 loop antibodies (*Figure 4a*). The accompanying data also indicated that inoculation with the plasmid cocktail from divergent clones of ENV allowed the co-expression of the divergent GP120 (*Figure 4d*). Antibody elicited with the plasmid cocktail was capable of reacting with divergent test peptides from AUG06c and CUG045 (*Figure 4d*). The titers generated by cocktail-inoculated mice were generally much higher vs immunization with single clones (*Figure 5*). These data do not exclude the possibility that the specificities and titers of the antibodies elicited in either single or multiple plasmid inoculation could change over time following additional boosts. However, our results appear to demonstrate the use of the CMVint-BL vector to facilitate the evaluation of a large number of the currently available HIV-1 candidate vaccines in a timely manner. Of similar interest was the observation that the V3 peptide from the heterologous clone MN and CUG045 stimulates proliferation of spleen cells (*Table 1*). In this instance DNA-mediated immunization with rgp120 of AUG06c appears to promote cellular immune response of relatively broad specificity.

In contrast to the immunogenic potential demonstrated for rgp120 of AUG06c and CUG045, results for the analogous molecule from the subtype D clone, DUG23c, were not as striking (*Figure 4c*). Yet the antigenicity of rgp120 peptide from CMV/DUG23c seemed comparable to, if not better than that of CMV/AUG06c or CMV/CUG045 (*Figure 3*). It should be noted that several attempts have been made to immunize mice with the plasmid clone from DUG23c and other subtype D isolates (not shown). None of the mice immunized with ENV plasmids from these clones has yet seroconverted when tested with the V3 loop PND peptide at 3 months post priming. These results may be subject to several interpretations: a defect in the chemical synthesis of the PND peptides from the subtype D virus could impair antibody binding in ELISA, or lack of a β-turn conformation in the V3 loop of DUG23c[5,7] could modulate its antigenicity. Conductivity traces have revealed no abnormalities in the synthesis of this peptide. DUG23c, like many other subtype D clones, has been shown to lack an antigenic β-turn conformation in the V3 loop[5,7]. Conceivably, the decreased immunogenic potential of the PND could serve to evade immune recognition of these strains *in vivo*. Further analysis of the antigenic properties of the D subtype of HIV-1 may be warranted.

REFERENCES

1 WHO Network for HIV Isolation and Characterization 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* 1994, **10**, 1327

2 Myers, G., Wain-Hobson, S., Korber, B., Smith, R.F., Berzofsky, J.A. and Pavlakis, G.N. Human Retroviruses and AIDS Database. A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences, 1993. Los Alamos National Laboratory, Los Alamos, NM

3 Janssens, W., Heyndrickx, L., Van de peer, Y. *et al.* Molecular phylogeny of part of the env gene of HIV-1 strains isolated in Cote d'Ivorie. *AIDS* 1994, **8,** 21

4 Nkengasong, J.N., Janssens, W., Heyndrickx, L. *et al.* Genotypic subtypes of HIV-1 in Cameroon. *AIDS* 1994, **8,** 1405

5 Pestano, G., Hosford, K., Spira, A. *et al.* Seroreactivity of analogous and distinct antigenic epitopes in gp120 expressed in HIV-1 subtypes A, B, C and D. *AIDS Res. Hum. Retroviruses* 1995, **11,** 589

6 Janssens, W., Heyndrickx, L., Fransen, K. *et al.* Genetic and phylogenetic analysis of env subtypes G and H in Central Africa. *AIDS Res. Hum. Retroviruses* 1994, **10,** 887

7 Pestano, G., Prince, A., Guyden, J., Ntambi, J., Atkin, A. and Boto, W.O. Independent divergences in the CD4 binding site and V3 loop encoded in two seroprevalent Ugandan HIV-1 clinical isolates. *J. AIDS* 1993, **6,** 872

8 Atkin, A., Pestano, G., Serwadda, D. *et al.* Phylogenetic and serological characterization of two Ugandan HIV-1 isolates. *AIDS Res. Hum. Retroviruses* 1993, **9,** 351

9 Sambrook, J., Fritsch, E.F. and Maniatis, T. Molecular Cloning. A Laboratory Manual, 2nd, 1989. Cold Spring Harbor Laboratory Press, Plainview, NY

10 Chapman, B.S., Thayer, R.M., Vincent, K.A. and Haigwood, N.L. Effects of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. *Nucleic Acids Res.* 1991, **19,** 3979

11 Rhodes, G., Dwaki, V., Abai, A.M. *et al.* Ginsberg, H.S. Brown, F. Chanock, R.M. Lerner, R.A. Vaccines '93 Modern Approaches to New Vaccines Including Prevention of AIDS, 1993, 137. Cold Spring Harbor Laboratory Press, Plainview, NY

12 Ulmer, J., Donnelly, J.J., Parker, S.E. *et al.* Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 1993, **259,** 1745

13 Foecking, M.N. and Hofstetter, H. Powerful and versatile enhancer-promoter unit for mammalian expression vectors. *Gene* 1986, **45,** 101

14 Sevarino, K.A., Felix, R., Banks, C.M. *et al.* Cell specific processing of preprosomatostatin in cultured neuroendocrine cells. *J. Biol. Chem.* 1987, **262,** 4987

15 Crockett, M.I., Bebbington, C.R. and Yarranton, G.T. The use of engineered E1A gene to transactivate the hCMV-MIE promoter in permanent CHO cell lines. *Nucleic Acids Res.* 1991, **19,** 319

16 Andersson, S., Davis, D.L., Dahlback, H., Jornvall, H. and Russell, D.W. Cloning, structure and expression of the mitochondrial cytochrome p-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.* 1989, **264,** 8222

17 Schmidt, E.V., Christoph, G., Zeller, R. and Leder, P. The cytomegalovirus enhancer: a pan-active control element in transgenic mice. *Mol. Cell. Biol.* 1990, **10,** 4406

18 Stephens, P.E. and Cockett, M.I. The construction of a highly efficient and versatile set of mammalian expression vectors. *Nucleic Acids Res.* 1989, **17,** 7110

19 Okudo, K., Bukawa, H., Hamajima, K. *et al.* Induction of potent humoral and cell-mediated immune responses following direct injection of DNA encoding the HIV type 1 env and rev gene products. *AIDS Res. Hum. Retroviruses* 1995, **11,** 933

20 Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976, **72,** 248

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

22 Wolff, J.A., Williams, P., Acsadi, G. *et al.* Conditions affecting direct gene transfer into rodent muscle *in vivo. Biotechniques* 1994, **11,** 474

23 Parker, J.M.R. and Hodges, R.S. Prediction of surface and interior regions in proteins—part 1: linear tripeptide sequences identify structural boundaries in proteins. *Peptide Res.* 1991, **4,** 347

24 Chou, P.Y. and Fasman, G.D. Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol.* 1978, **47,** 45

25 Merrifield, R. Solid-phase peptide: the synthesis of a tetra-peptide. *J. Am. Chem. Soc.* 1963, **85,** 2149

26 Ahlers, J.D., Pendleton, C.D., Dunlop, N., Minassian, A., Nara, P.L. and Berzofsky, J.A. Construction of an HIV-1 peptide vaccine containing a multideterminant helper peptide linked to a V3 loop peptide 18 inducing strong neutralizing antibody responses in mice of multiple MHC haplotypes after two immunizations. *J. Immunol.* 1993, **150,** 5647

27 Aldovini, A. and Walker, B.D. Techniques in HIV Research, 1990, 213. Stockton Press, UK

28 Levy, J.A. HIV and the Pathogenesis of AIDS, 1994, 8. American Society of Microbiology Press, Washington, DC

29 Wolf, H., Modrow, S., Motz, M., Jameson, B.A., Hermann, G. and Fortsch, B. An integrated family of amino acid sequence analysis programs. *CABIOS* 1988, **4,** 187

30 Modrow, S., Hahn, B.H., Shaw, G.M., Gallo, R.C., Wong-Staal, F. and Wolf, H. Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions. *J. Virol.* 1987, **61,** 570

31 Wang, B., Ugen, K.E., Srikantan, V. *et al.* Gene inoculation generates immune responses against human immunodeficiency virus type 1. *Proc. Natl Acad. Sci. USA* 1993, **90,** 4156

32 Robinson, H.L., Ginsberg, H.S., Brown, F., Chanock, R.M. and Lerner, R.A. Vaccines '93 Modern Approaches to New Vaccines Including Prevention of AIDS, 1993, 311. Cold Spring Harbor Laboratory Press, Plainview, NY