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Structure-antigenicity of the V3 region of SIVmac envelope glycoprotein

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The objective of this study was to analyze the immunogenicity and antigenicity of the V3 domain (Cys313–Cys346) of the external envelope glycoprotein gp125 of SIVmac251. The corresponding peptide was synthesized and characterized as linear and cyclic peptides. Our results showed that this region, as for HIV-1, contained an immunodominant epitope. The antigenicity was similar for the linear and cyclic peptides when tested against a panel of 15 sera from SIV infected macaques. Similarly, both peptide structures presented similar immunogenicity as shown by the characterization of the anti-peptide antibodies produced in rabbits against the cyclic and linear forms. But, unexpectedly, the antibodies produced against linear peptides recognized with a relatively higher intensity the native envelope gp140 than those produced against the cyclic structure. Furthermore, we showed that these antibodies recognized better the deglycosylated form of the glycoprotein. But, in contrast to the neutralizing activity obtained with anti-V3 peptides from HIV-1, no antiviral activity was obtained with antibodies generated against linear or cyclic SIVmac V3 peptides. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antibodies; peptide; SIV; V3; HIV

Introduction

Simian immunodeficiency virus (SIV) is a non-human primate lentivirus related to the retroviridae family. It was initially isolated from a captive rhesus monkey [1]. Like human immunodeficiency viruses, HIV-1 and HIV-2, this virus induces an AIDS-like disease only in non-natural monkey hosts, such as Asian monkeys. Although naturally infected, African monkeys develop only an asymptomatic chronic infection [2,3]. HIV and SIV genomes present similar organization, except for the presence of vpx and Vpu genes in SIV and HIV genomes respectively [4]. SIV also shares several additional properties with HIV including tropism for CD4 positive cells, oligomeric structure of the surface glycoprotein (gpSU) and the transmembrane glycoprotein (gpTM), which are both assembled as trimers on the surface of the viral particles. The physiopathology of the infection is also comparable. All these properties make SIV/macaque a valuable model for evaluating candidate vaccines and antiretroviral drugs.

In contrast to HIV-1, the native form of SIV precursor gp140 seems to be essential for the generation of appreciable titers of neutralizing antibodies. Denatured gpSU or synthetic peptides corresponding to that of the HIV-1 principal neutralizing determinant, also named the V3 region, are unable to induce or adsorb measurable amounts of neutralizing antibodies [5]. In line with the latter report, the same group has also shown that SIV-infected monkeys produce neutralizing antibodies that continue to interact with a 45 kDa fragment that includes both V3 and V4 variable loops of SIV gpSU [6]. However, several groups have also described linear neutralizing epitopes in the SIV V2 region between amino acids (aa) 170 and 190 [7] and in the V4 loop as shown by the capacity of this peptide to adsorb neutralizing antibodies from the sera of SIV infected macaques [8]. This suggests the presence of a linear epitope of neutralization within this region. In accordance with these results, our group produced and characterized monoclonal antibodies (Mab1B9) which recognized a linear epitope in the V4 region (aa 411–430) of the gpSU and neutralized SIV replication [9].

Several lines of evidence have also shown the crucial role of the conformational structure of gp120 from HIV-1 in the induction of protective immune responses. At least four categories of neutralizing antibodies have been purified from HIV-1 infected human sera, including those directed against the linear epitope within the V3 region, the conformational CD4 binding site, the carbohydrate moieties and the quaternary epitopes present only in the oligomeric structure of gp120 [10]. In agreement with the production of neutralizing antibodies against the oligomeric form of the viral envelope glycoproteins, it has been shown that the structural antigenicity of the monomeric form of HIV-1 gp120 differs from that of the oligomeric form [11], and that 70% of HIV-1 sera antibodies bind to epitopes present only in oligomeric envelope proteins [12]. The importance of the quaternary structure of the envelope glycoprotein in the generation of neutralizing antibodies has been confirmed by Lüke *et al*. [13], who showed that macaques immunized with SIV envelope glycoprotein oligomers were protected against SIVmac32H.

The highly conserved cysteine positions in HIV-1, HIV-2 and SIV SU suggest a considerable structural role for this protein [14]. Moreover, loop regions, which by virtue of their location, are prime candidates for the induction of specific antibodies, represent the major targets of the products of the immune responses. As a consequence, the major antigenic sites are located within these variable regions. Immunogenicity and antigenicity of these regions were largely analyzed by using anti-peptide

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Journal of PeptideScience

antibodies. The advantage of this approach is that it permits to obtain antibodies that have predetermined specificity, thus allowing some manipulation of the specificity of the immune response. Unfortunately, antibodies produced against synthetic peptides often do not recognize native protein or the related whole viral particle, and thus have only a limited application as candidate vaccines when used as linear peptides. Some reasons for this limitation are related to the conformation adopted by the synthetic peptides. In fact, such peptides can exist in two forms, PN and PR. Whereas PN is the conformation similar to that the peptide would possess in the native protein, PR is the ensemble of unordered or denatured forms of the peptide. It has been estimated that only $1/10^4$ to $1/10^6$ of linear peptides have the correct PN conformation [15–17]. Several methods have been used to enhance the effectiveness of synthetic peptides with varying degrees of success, including circularization of peptides [18–21], multiple repetitions of a protective epitope [22,23] fusion of T cell and B cell epitopes [24] or engineered peptides in which substitution residues stabilize their secondary structure [19,25].

In the present study, we evaluated the antigenicity and the capacity of linear and cyclic peptides modelling the V3 region of SIV envelope glycoprotein to induce neutralizing antibodies. To this end, the peptide corresponding to the V3 region (aa 313–346) was chemically synthesized by the solid phase method and purified by chromatography to homogeneity. Cyclic Cys313–Cys346 peptide was obtained after reoxidation and further HPLC purification. Linear and cyclic peptides were used as immunogens to produce specific antibodies in rabbits and as antigens to test a panel of 15 sera obtained from SIV infected macaques. Our results showed that the V3 peptide of SIV, like its homologue in HIV-1, corresponds to an immunodominant region (a domain which is highly recognized by the product of the immune response), as shown by its antigenicity against 100% of the sera (15/15) from SIV infected macaques tested. However, both linear and cyclic V3 peptides of SIV are unable to induce neutralizing antibodies such as V3 of HIV-1, thus suggesting a different functional role for V3 of SIV.

Material and Methods

Peptide Synthesis

The 34 amino acid peptide mimicking the V3 region derived from SIVmac251 envelope glycoprotein was synthesized by the solid phase method using protected Fmoc amino acids [26]. Peptide synthesis was carried out automatically on an Applied Biosystem peptide synthesizer. 0.1 mmol of HMP resin (4-hydroxymethylphenoxymethyl resin) (Novabiochem) was used. An additional alanine residue was introduced at the *C*-terminus of the peptide in order to enhance the efficiency of peptide-resin cleavage. Fmoc-Ala-OH preactivated with TBTU/HOBt/DIEA(1/1/1.5) in DMF was coupled. After 2 h, the resin was washed with DMF (3 × 1 min) and DCM (3 × 1 min) and the free amino groups were capped with acetic anhydride/DIEA/DMF (3 : 1 : 16) (2 × 30 min). The resin was washed with DMF (3 × 1 min) and the Fmoc group was removed with 50% piperidin/DMF (3 × 5 min). Rink amide linker was coupled after preactivation with TBTU/HOBt/DIEA (1 : 1 : 1.5) in DMF. Fmoc/tBu protected amino acids (Iris Biotech GmbH) were incorporated using standard procedures and TBTU/HOBt/DIEA as coupling agents. Fmoc cysteine amino acids were used with unprotected SH. After final deprotection, peptides were acetylated by anhydride acetic treatment (40 eq., 9 eq. of DIEA, DMF). Concomitant side-chain deprotection and cleavage was performed using 5 ml of a mixture of trifluoroacetic acid (TFA)/H2O/triisopropylsilane/ethanedithiol (92 : 4 : 2 : 2 v/v/v/v) at 0 °C for 30 min and at room temperature for 1.5 h. TFA was removed by evaporation and the crude peptide was precipitated with *tert*-butyl methyl ether.

The crude peptide was purified by RP-HPLC on an aquapore RPC30C18 column (22.0 × 1.0 cm). Peptide was eluted with a 30 min gradient from 30 to 80% of buffer B (Buffer A = 0.1%TFA; Buffer B = 60%CH3CN/40%H2O/0.1%TFA). The purified peptide was further characterized by aa content determination after hydrolysis (6N HCl, 1 h at 150 °C or 20 h at 110 °C) analytical HPLC, and MALDI–TOF mass spectrometry. The homogeneity of the purified peptide was >95% as confirmed by the analytical methods.

Reduction and Alkylation of V3 Peptide

Synthetic peptide was first reduced in 1 ml of buffer containing 0.25 M Tris-base, 6 M guanidine, 14 mM EDTA pH 8.6 with 60-fold excess of DL-dithiothreitol (DTT from Sigma) over sulfhydryl groups. The solution was flushed with nitrogen and maintained at 40 °C for 20 h in the dark. Next, a twofold excess of iodoacetamide over DTT was added for 20 min at room temperature in the dark to prevent secondary reactions. Finally, 5 µl of β-mercaptoethanol was added to stop the reaction. The alkylated peptide was desalted by HPLC, using a C18 column and an H2O-acetonetrile gradient system containing 0.1% trifluroacetic acid.

Preparation of Cyclic Peptide

We first verified the reduction state of the sulfhydryl groups in the peptide by using the Ellman test (Ellman 1959). With this test, we found that about 80% of the peptide presented SH free groups. Total reduction of the peptide was performed by using 1.1 excess of tributylphosphine (Merck) in 2 ml of a mixture of equal volume of 0.1 M Tris–HCl pH 8.0 and *n*-isopropanol (Merck). Incubation was conducted for 20 h under nitrogen and in darkness. The reduction medium was then diluted in 200 ml of 0.1 M ammonium acetate buffer pH 8.0 and subjected to magnetic stirring for 72 h in contact with air. After lyophilisation, the cyclic peptide was purified by HPLC using a C18 column and an H2O-acetonetrile gradient system containing 0.1% trifluroacetic acid. The purity of the cyclic peptide was confirmed by mass spectrometry.

Immunization of Rabbits

New Zealand white rabbits were immunized intradermally with 200 µg of linear or cyclic peptide in combination with complete Freund's adjuvant. The rabbits were boosted with three subcutaneous injections with 200 µg of each peptide in combination with incomplete Freund's adjuvant at 4-week intervals. Ten days after each injection, blood samples were taken and analyzed for the presence of antipeptide antibodies using an ELISA test. Two rabbits were used for immunization with each peptide.

Enzyme-linked Immunosorbent Assay

The wells of 96-well microtiter plates (Nunc, Rotskild, Denmark) were coated with either linear or cyclic peptide (500 ng in 50 µl of

PBS, pH 7.4) and left overnight at 4 °C. After saturation with 200 µl of PBS supplemented with 5% of non-fat milk for 1 h at 37 °C and washing with 0.2% Tween-20 in PBS, 50 µl of different serum dilutions were added and incubated for 2 h at 37 °C. After rinsing, 50 µl of 1/1000 of horseradish peroxidase labelled anti-rabbit (Dako, Glostrup, Denmark) was added during 1 h at 37 °C. After further rinsing, 100 µl of orthophenylenediamine was added and the samples incubated for 30 min in the dark at room temperature. The reaction was stopped by adding 25 µl of 4N sulfuric acid and the 490/600 nm absorbance ratio was determined. Serum titer was defined as the highest dilution which gave a positive signal.

Recombinant Soluble gp140

Recombinant soluble gp140 of SIVmac251 [27] was obtained from Pasteur Mérieux Serum et Vaccin (Val de Reuil, France). Briefly, the env-coding sequence of gp140 was derived from SIVmac251 envelope precursor protein by deletion of the potential transmembrane segment (Ile693-Leu716). The potential cleavage site was also mutated to prevent processing of the SU and TM proteins. Recombinant vaccinia-virus-infected BHK cells produced gp140, which was then purified from the supernatant by lectin affinity chromatography and gel filtration.

Radio-immunoassay

SIVmac251 gp140 was iodinated as described previously [28]. RIA was performed as follows: 50 µl of ^{125}I-gp140 (10^5 cpm) was incubated with various serum dilutions for 2 h at 37 °C. The complexes formed between ^{125}Igp140 and anti-peptide antibodies were immunoprecipitated during 1 h at room temperature with 100 µl of Protein A-Sepharose (1.5 g/50 ml) in PBS, pH 7.4 containing 0.5% BSA and 0.05% NaN3. After washing twice with the same buffer containing 0.05% Tween-20, bound radioactivity was counted in a gamma counter.

Deglycosylation of ^{125}I-gp140

^{125}Igp140 (10^7 cpm) in PBS buffer pH 7.4 was incubated at 37 °C with endoglycosidase F/N-glycosidase F (400 mU) (Boehringer Mannheim Biochemicals, Mannheim, Germany). The glycosidase enzyme was added in three aliquots at 6-h intervals. The reaction of deglycosylation was controlled by SDS-PAGE and autoradiography.

Cells and Virus

CEMx174 cells, H9 cells chronically infected with SIVmac251, uninfected C8166 cells and human peripheral blood cells (PBL) were cultured in RPMI-1640 medium (Eurobio, Les Ulis, France) supplemented with 10% of foetal calf serum (ATGC, Orleans, France), 2 mM glutamine, and antibiotics (100 IU penicillin and 100 mg/streptomycin) (Eurobio, Les Ulis, France) in a 5% CO_2 humidified atmosphere. PBL from healthy human donors were separated by Ficoll-Hypaque, stimulated with phytohemagglutinin P (PHA-P from Difco laboratories, Detroit, Michigan, USA), diluted at 10 µg/ml for 72 h at 37 °C, infected with SIVmac251 and maintained in a culture medium containing 20 U/ml of IL-2.

Inhibition of Syncytium Formation and Virus Neutralization Assays

Briefly, 10^4 H9 or PBL chronically infected cells were incubated with various dilutions (1/10 to 1/1000) of anti-peptide sera previously inactivated by heating for 20 min at 56 °C. After 1 h at 37 °C, 4×10^4 uninfected C8166 cells were added and cocultured for 18 h at 37 °C. The number of syncytia was then scored under a microscope. In each experiment, the effect of the neutralizing anti-CD4 antibody Leu3a (Becton-Dickenson, San Diego, USA) was used at 2.5 µg/ml, in parallel as a positive control for total inhibition of cell fusion.

Neutralizing activities of linear and cyclic anti-peptide antibodies were tested on human PBL and CEMx174 as targets for SIVmac251. The neutralization assay (titer reduction assay) was performed as described in [29].

Labelling by Anti-peptide Antibodies of SIV Envelope Bound to CEM cells CD4

Soluble SIV envelope glycoprotein gp140 was incubated for 1 h at 37 °C with various dilutions of anti-V3 peptide. The preformed complex gp140-antipeptide was incubated with 10^6 CEM cells for 30 min at 37 °C in 20 µl of PBS buffer pH 7.4 supplemented with 0.5% BSA and 0.05% NaN_3. After two washes, cells were incubated again for 30 mn at 4 °C with 1/25 swine anti-rabbit Ig complexed with biotin (Amersham). Cells were then washed and resuspended in 25 µl of 1/25 streptavidin–phycoerythrin (Becton-Dickinson) in the cold. After two more washes, cells were resuspended in 500 µl of PBS buffer with 1% paraformaldehyde. Membrane fluorescence intensity was measured with a FACS analyser (Becton-Dickinson).

Results

The aim of our study was, on one hand to determine whether the structure of the V3 domain (Cys313–Cys346) of the external envelope glycoprotein gp125 of SIVmac251 may influence its antigenicity, immunogenicity, and its capacity to induce neutralizing antibodies and on the other hand to analyze the specificity of antipeptide antibodies to recognize native and deglycosylated envelope glycoprotein.

Characterization of Linear and Cyclic Peptides

Peptide mimicking the V3 region of the external envelope glycoprotein of SIVmac251 (Figure 1) was synthesized using a solid phase method with Fmoc amino acids strategy. The assembled peptide, containing unprotected SH of cysteines, was purified to homogeneity by HPLC (Figure 2a). After SH quantification by the Ellman test, more than 80% of the peptide was shown to be present in reduced form. The small peak (Figure 2a) corresponds to the cyclic peptide. The homogeneity of the peptide was further confirmed by amino acid composition and molecular weight determination by MALDI–TOF. Purified peptide was subjected to an additional reduction followed by SH alkylation (Figure 2b). Despite this modification, the alkylated peptide was eluted at a similar retention time as its reduced form (Figure 2a and b). However, the aim of this step was to prevent the formation of cyclic or oligomeric peptides during the use of this peptide and, thus, to be sure that all the biological conclusions obtained with this structure of the peptide were really related to the linear form.

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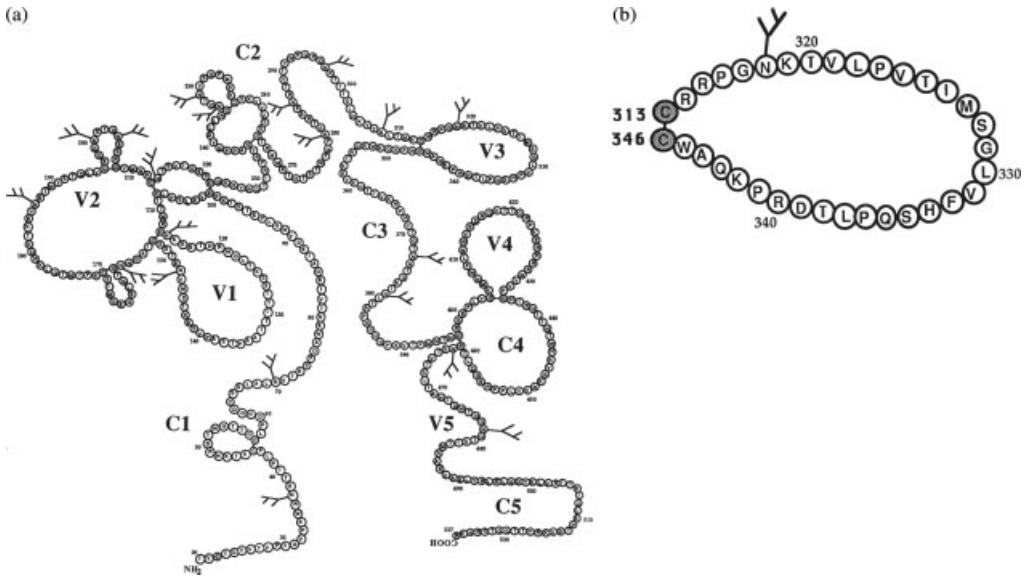

Figure 1. (a) Hypothetical assignment of disulfide bonds for SVImac251 external envelope glycoprotein as suggested by Hoxie *et al*. 1991. The disulfide bonds are numbered from 1 to 11. Glycosylation sites are shown in the sequence by branched glycosyl derivatives. The conserved (C1–C5) and variable (V1–V5) regions are indicated. (b) Presentation of the secondary structure of the V3 region of SIVmac251 (BK28 clone).

To obtain the cyclic form, previously reduced peptide with a slight excess of tributylphosphine was diluted in 0.1 ammonium acetate buffer at pH 8.0, and air oxidation was conducted in a large volume for 48 h. The kinetics of oxidation was monitored by HPLC (Figure 3). Before oxidation, the chromatogram exhibit one peak (Figure 3a). After 12 h, three peaks corresponding to two forms of cyclic and linear peptides can be identified (Figure 3b). The peak eluted with an intermediate retention time corresponds to the linear form (Figure 3b). After 48 h of oxidation, this peak completely disappeared, suggesting total oxidation (Figure 3c). The position of the peak corresponding to the linear peptide was further confirmed by adding an exogenous aliquot of this peptide to the purified product obtained after 48 h of oxidation (Figure 4, peak 2). The corresponding contents of each peak were further analyzed by MALDI–TOF. This characterization showed that the determined MW of the peak 3 (MW = 3919.6 Da) was in agreement with the expected MW of the correct cyclic peptide, while that of the peak 1 (MW = 3935.6 Da) seemed to correspond to V3 peptide with an oxidized methionine as suggested by its upper MW of 16 Da (data not shown).

Antigenicity of Linear and Cyclic V3 Peptides

Linear and cyclic peptides were used as antigens and tested by ELISA, in the same conditions, against a panel of 15 sera obtained from macaques infected with SIVmac251 (clone BK28). Preimmune sera, obtained from the same macaques, were used as controls. The results obtained are the following: (i) A significant reactivity against both linear and cyclic peptides was observed with all the sera tested. This reactivity seems to be specific as shown by the absence of positive signals with preimmune sera (Figure 5). This result clearly demonstrated the presence of an immunodominant antigenic determinant in the V3 region of the SU of SIV as shown by the immunoreactivity of this peptide with 100% (15/15) of the sera from SIV infected macaques. (ii) Among the 15 sera tested, 10 seemed to recognize the cyclic form of the V3 peptide better than the linear form, while sera from macaques C305 and C298 recognized the linear peptide better (Figure 5). But, overall, the antigenicity between linear and cyclic peptides remained comparable as shown in Figure 5. It is interesting to note that these results are comparable to those previously reported for the V3 region of HIV-1 [30]. However, in contrast to HIV-1, the sequence of the V3 region in SIV seems to be less variable, as reported by Burns and Desrosiers [31].

Immunogenicity and Antigenicity of Linear and Cyclic V3 Peptides

Sera produced against linear and cyclic V3 peptides were obtained with high titers, about $1/10^7$, when tested by ELISA against adsorbed peptides. Interestingly, the anti-peptide antibodies produced against the linear V3 peptide recognized the linear structure with the same intensity as it recognized the cyclic one. Antibodies produced against the V3 loop exhibited a similar spectrum of reactivity against both structures of V3 peptides (Figure 6). This result suggests that cyclization did not introduce sufficient structural constraints to generate antibodies able to differentiate between the two forms (linear and cyclic), at least when the antigenicity was tested against peptides in ELISA.

Reactivity of Anti-peptide Antibodies against Native Envelope Glycoprotein

In order to evaluate the binding activity of anti-peptide antibodies against native gp140 of SIV, anti-peptide antibodies were tested in a liquid phase RIA, which enabled antigen–antibody interactions to be measured in non-denaturing conditions. The results obtained showed that both linear and cyclic peptides were able to generate antibodies that recognized native envelope glycoprotein (Figure 7). Antibodies produced against linear peptide were produced with a titer ten times higher than those obtained against cyclic peptide. This result showed that, in contrast to the results obtained in the antigenicity tests and contrary to the hypothesis advanced, cyclization of the peptide contributed negatively to the generation of antibodies that recognize native

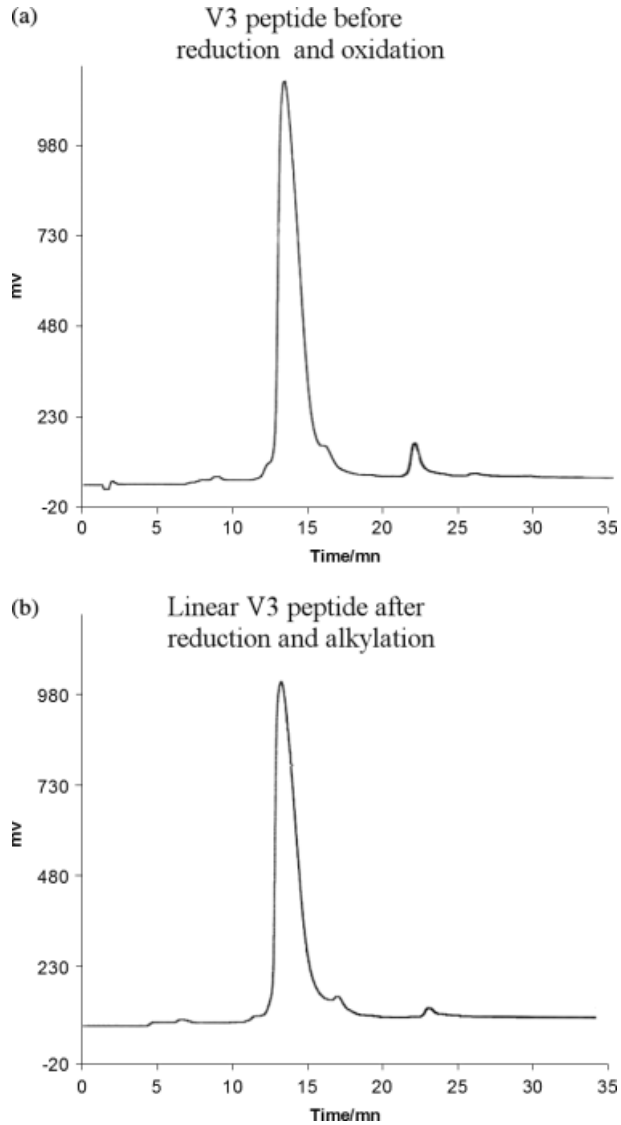

Figure 2. Analytical high performance liquid chromatography of linear V3 as obtained after its purification on a C18 column (a) V3 peptide before alkylation of free SH groups. (b) V3 peptide after reduction and alkylation of free SH groups. An aquapore C18 RP300 column (24 × 4.6 mm) was used. Elution was performed with 0.1% TFA system buffer with a 25 min linear gradient from 10 to 100% of buffer A. Buffer A = 0.1% TFA, buffer B = 60% CH_3CN/40% H_2O/0.1% TFA.

envelope glycoprotein. The better reactivity observed with anti-linear peptide may be also explained by its better flexibility, which may better mimic native immunogenic determinants than the more rigid cyclic structure.

Because V3 contains a conserved *N*-linked glycosylation site, we tested the effect of carbohydrate moieties on antigen-antibody interactions. To this end, deglycosylated ^{125}Igp140 was tested in RIA against antibodies produced against cyclic and linear peptides. The results, depicted in Figure 7, showed an enhancement in the reactivity of anti-peptide against the deglycosylated envelope glycoprotein form.

Neutralization Activity of Anti-peptide Antibodies

The neutralization capacity of anti-peptide antibodies produced against linear and cyclic peptides was tested in two assays. The first was syncytium inhibition activity *in vitro*. Chronically infected H9 or human peripheral blood lymphocyte cells were co cultured with uninfected C8166 cells in the presence of various dilutions of anti-peptide sera, or with anti-CD4 Leu3a as positive control. As expected, anti-CD4 Leu3a completely inhibited cell fusion, while none of the anti-peptide antibodies inhibited syncytia formation.

We also carried out a cell-free virus neutralization assay against SIVmac251. The assay was based on the capacity of anti-peptide antibodies to inhibit virus infection of CEMx174 and PBL cells. None of the antibodies produced against linear or cyclic peptides displayed significant neutralizing activity against SIVmac251 even at low dilutions such as 1/20 (Table 1). These results are in agreement with the fact that anti-V3 antibodies were unable to prevent binding of soluble recombinant gp140 to CD4 receptor on the CEM cells (Figure 8). This indicates that the V3 region remained accessible to antibody binding after gp-CD4 complex formation. As positive control we showed that when tested in the same conditions, the neutralizing monoclonal antibody TB10, directed against gp140, became unable to label SIV envelope gp140 previously complexed to cell surface CD4 receptor (Figure 8).

Discussion

SIV shares several common properties with HIV-1 including tropism for receptors CD4 and CCR5/CXCR4, and the establishment of an AIDS-like disease [32,33]. Thus, the SIV/macaque model is valuable for pathogenesis studies and vaccine development. As for HIV-1, the envelope glycoprotein of SIV has four variable loops formed by highly conserved cysteines. This common conserved secondary structure of the gpSU seems to play a crucial role in the conservation of the bioactive conformation of this protein. Reduction of preformed S-S bridges totally abolishes the capacity of SIV and HIV envelope glycoproteins to interact with CD4 receptors [34] and with some neutralizing antibodies that recognize conformational epitopes [35,36].

Like the conserved regions, variable regions also play an essential role in the first steps of a viral cycle. For example, following gp-CD4 interaction, V1 and V2 domains are rearranged in order to make a coreceptor binding site accessible [37–39]. Similarly, some conserved residues located at the tip and the stem of the V3 loop of HIV-1 have been reported to be important for gp-coreceptor interactions and virus entry [40–42]. Many studies have also suggested that glycosylation in VI, V2 and V3 regions seems to be essential in the tropism of the viruses [43,44] and in the accessibility of neutralizing antibodies to the receptor binding domains [45]. However, studies reported by our group have demonstrated that glycan moieties of HIV-1, HIV-2 and SIV envelope glycoproteins play only a limited role as shown by the capacity of totally deglycosylated gpSU of these viruses to continue to interact with CD4 [28,46,47]. Furthermore, we have shown that deglycosylated HIV-1 viral particles conserve some of their infectious power [46]. This apparent discrepancy between our studies and those of others [48–50] may be explained by the fact that carbohydrate moieties may be essential for the acquisition of the native conformation of the envelope glycoproteins.

In addition, these variable regions, which are more accessible at the surface of the proteins, are more immunogenic than the less accessible conserved domains (C1–C5). In consequence the following observations are made: (i) Variable regions are further targeted by non-efficient neutralizing antibodies, also defined

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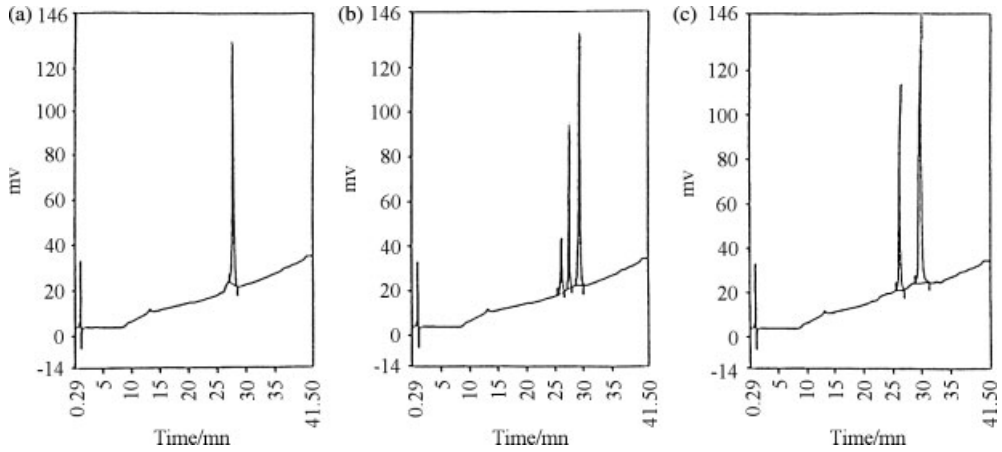

Figure 3. Kinetics of V3 peptide oxidation followed by HPLC analysis. Conditions of elution are the same as in Figure 2. The content of each peak was collected and analyzed by MALDI–TOF. (a) time = 0 min, (b) time = 12 h of oxidation, (c) time = 48 h of oxidation.

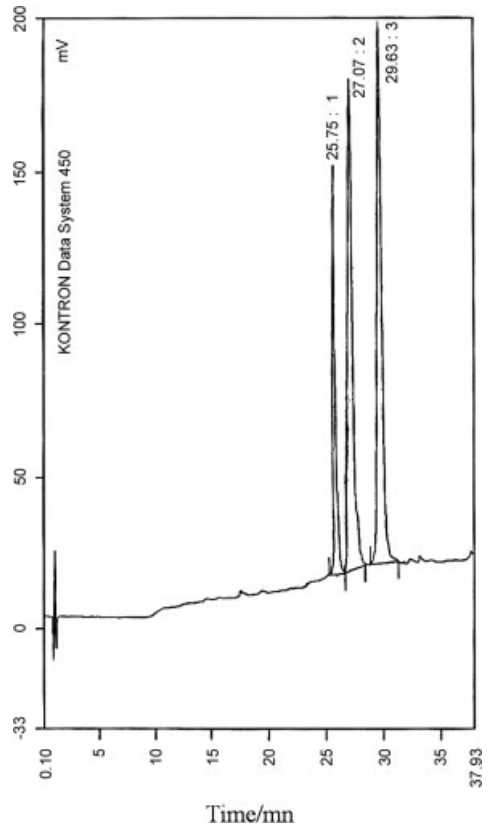

Figure 4. V3 peptide obtained after 48 h of oxidation was collected and added to an aliquot of totally linear peptide and this mixture was analyzed in the same conditions by HPLC. The analysis showed three separate structures. As expected, the linear peptide was eluted with the intermediate retention time peak 2. The peak 3 corresponds to the cyclised peptide and the peak 1 to the cyclised with an oxidized methionine. Conditions of elution were the same as for Figures 2 and 3 except that peptides were eluted with a 30 min gradient from 30 to 80% of buffer B on a RPC30 C18 column (22.0 × 1 cm).

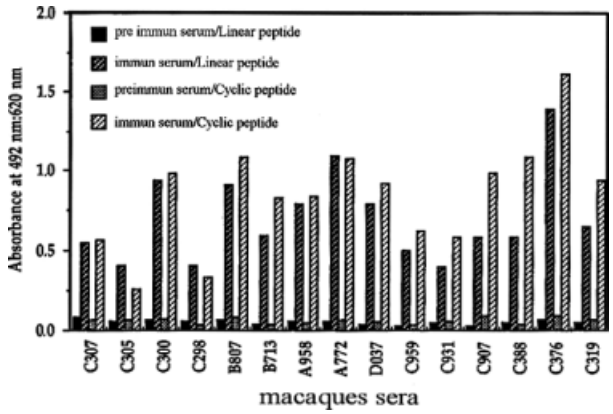

Figure 5. Comparative analysis by ELISA of the antigenicity of linear and cyclic V3 peptides. Immune and pre-immune sera of 15 SIV infected macaques were tested at 1/500 dilution.

as inhibitors of neutralizing antibodies. In fact, such antibodies continue to coexist in the sera of HIV infected patients, in the presence of the infectious viruses. (ii) Also, these variable regions may act by occluding the accessibility of conserved regions and thus reducing their immunogenicity and may prevent the binding of neutralizing antibodies, even if they are present, to these regions. The potential of the generation of such neutralizing antibodies is consistent with the reported neutralizing monoclonal antibodies directed against conserved peptidic regions such as b12 and 2F5 or against conserved glycanic domains such as human monoclonal antibodies 2G12 [51–53].

Several reports emphasize the importance of glycosylation at the level of variable regions on the generation of neutralizing antibodies. For example, Reitter *et al*. [54] showed that some mutations at the potential glycosylation sites within V1 and V2 regions may generate infectious viruses able to generate a greater amount of neutralizing antibodies and also neutralize the wild type virus. Inversely, it has been reported that some mutations, consisting of a deletion of 100 amino acids, which abolish glycosylation sites in the gpTM ectodomain of SIVmac239 lead to the obtention of replicative viruses which become sensitive to neutralization by sera produced against wild type virus [55]. More recently, it has been reported that deletion of an N-glycosylation site in the V1 region may influence the specificity of the produced anti-V3 antibodies [56]. Taken together, these results underline the importance of the three-dimensional structure of the V3 loop beyond its secondary structure, and the structure of carbohydrate chains located in V1, V2 and V3 loops. In a previous work, we showed that desialylated gp160 of HIV-1,

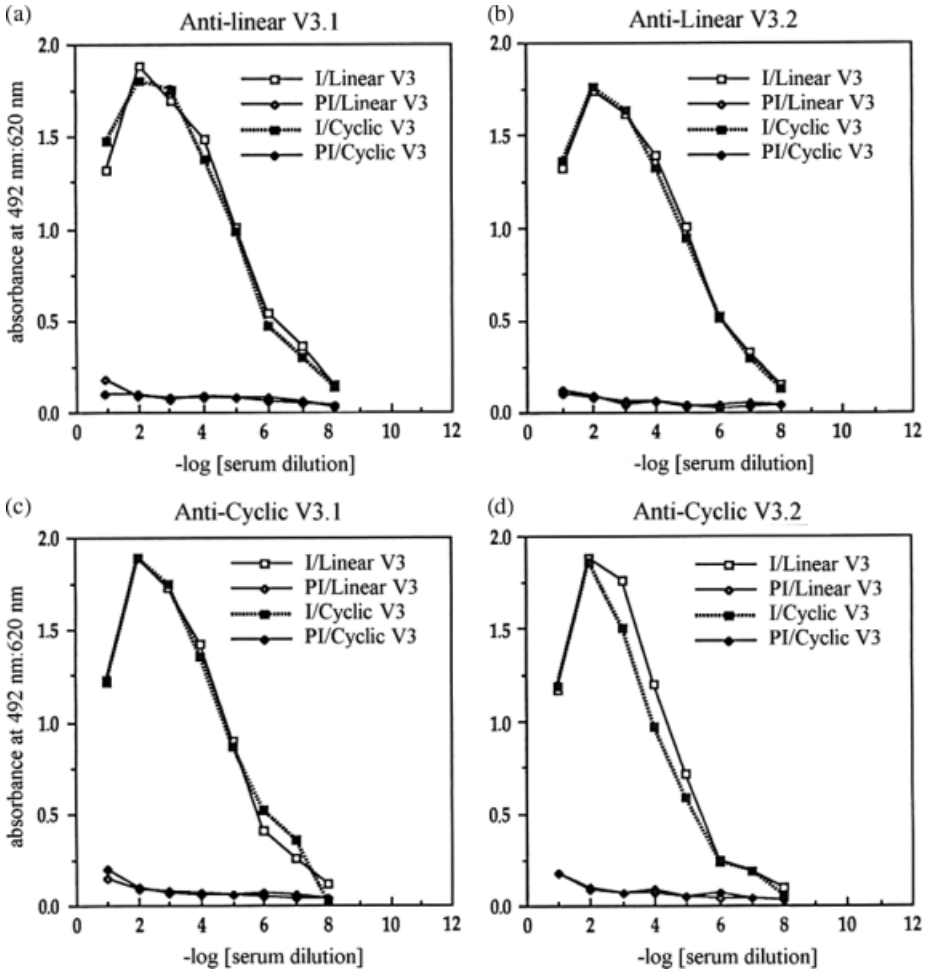

Figure 6. Titration by ELISA of anti-peptide sera (I) or pre-immune sera (PI) tested at various dilutions (1/10 to $1/10^8$) against various coated linear peptides from (a) rabbit 1(V3.1) and (b) rabbit 2(V3.2) or cyclic peptides from (c) rabbit 1 and (d) rabbit 2.

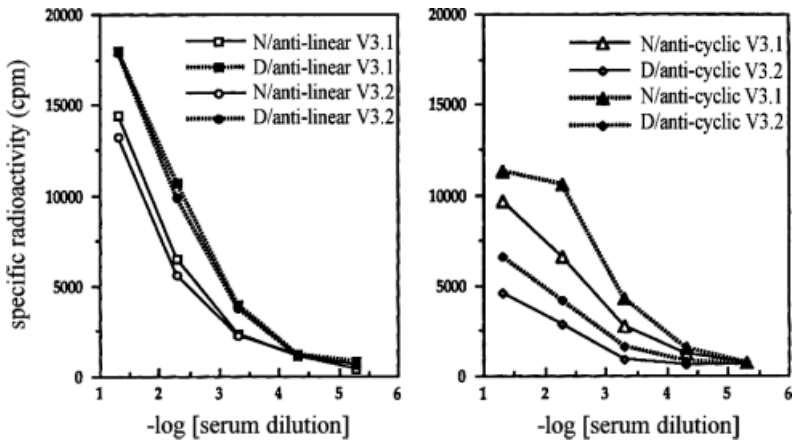

Figure 7. Analysis by radioimmunoassay of the reactivity of anti-linear and anti-cyclic anti-peptide antibodies. Native (N) or deglycosylated (D) ^{125}I gp140 from SIVmac251 was tested against various dilutions of immune sera (I) (1/10 to $1/10^6$) from rabbit 1(V3.1) or rabbit 2 (V3.2). Antigen-antibody complexes were immunoprecipitated by Protein A-sepharose. Pre-immune sera (PI) were used as controls.

obtained after neuraminidase treatment, generated antibodies with a broad spectrum of reactivity, as shown by their capacity to cross react with HIV-2 glycoproteins [57].

Antibodies produced against linear and cyclic synthetic V3 peptide (Cys313–Cys346) were tested for their reactivities against native and totally deglycosylated envelope protein. The results obtained showed that anti-linear and anti-cyclic anti-peptide antibodies recognized linear and cyclic peptides similarly when tested by ELISA. These results suggest two interpretations: (i) cyclization of the peptide has no effect on its immunogenicity,

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Table 1. Activity of the different antibodies

Antibodies	Concentration	Titer neutralization	Inhibition gp-CD4	Syncitia inhibition
Anti Linear V3 peptide	1/20	–	–	–
	1/100	–	–	–
Anti Cyclic V3 peptide	1/20	–	–	–
	1/100	–	–	–
Anti-Leu3a	2,5 µg/ml	+	+	+

or (ii) various sub-populations of antibodies, with different specificities, are present in the polyclonal antipeptide sera prepared against each peptide structure. This hypothesis is in line with the report showing the presence of an additional conserved neutralizing epitope around the GPGR sequence located at the tip of the V3 of HIV-1 [58].

Both anti-peptide antibodies, produced against linear or cyclic peptides, recognized the native envelope glycoprotein when tested in a liquid RIA. However, contrary to the hypothesis advanced, our data showed that anti-linear peptide antibodies recognized native envelope glycoprotein with a relatively better titer, about ten times higher, than those produced against cyclic peptide. These results suggest that the cyclization of the peptide is not sufficient to induce a more native-like structure or that linear peptide mimics this native-like domain better. However, in this work we showed that cyclic and linear peptides were recognized by all the sera (15/15) with a comparable antigenicity, confirming the presence of an immunodominant antigenic site in this region as also described for HIV-1 and HIV-2 [59,60]. Interestingly, our results showed that antipeptide antibodies recognized better the deglycosylated form of the envelope glycoprotein. In addition our results clearly demonstrated the role of glycan moieties in interfering negatively with the binding of antibodies to their corresponding antigenic sites. This is one mechanism by which infectious agents, including viruses, can escape immune system defences.

Despite the presence of anti-peptide antibodies able to recognize native envelope glycoprotein, these antibodies were unable to inhibit syncytium formation in coculture of infected and non-infected cells. In accordance with the latter result, these anti-peptide antibodies also failed to inhibit SIVmac251 replication in primary blood lymphocytes. These results agree with those reported for V3 of HIV-2 by various groups [61,62] including ours [63]. However, using monoclonal or anti-peptide antibody approaches, several neutralizing antibodies have been described and the recognized epitopes of some of them identified in the envelope glycoprotein sequence. Accordingly, linear neutralizing antigenic sites including epitopes in V1 [7,63–65], V4 [6,9] and the ectodomain of gp 41 [6,9,65,66] have been described. But many conflicting results have been reported for the capacity of the V3 region to generate neutralizing antibodies [5,29,67].

In our study, we showed that both linear and cyclic V3 peptides exhibited a similar antigenicity, suggesting the presence of an

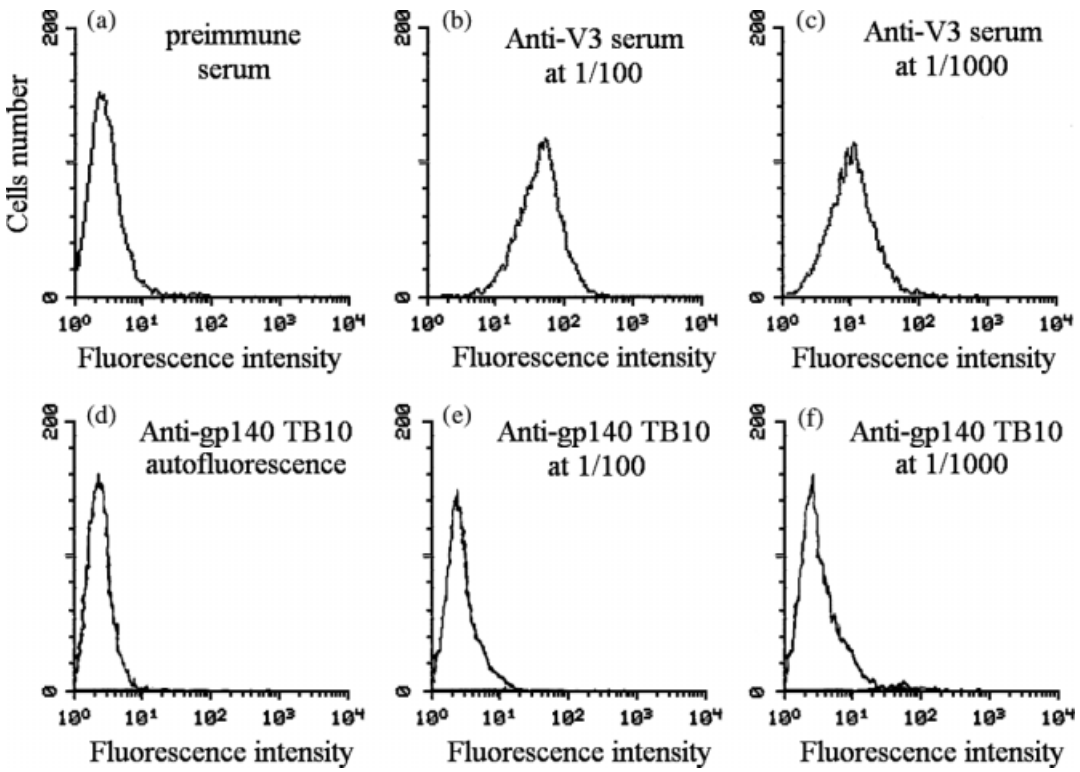

Figure 8. Labelling of soluble recombiant SIV gp140 binding to CD4+ CEM cells with anti-V3 antibodies. For specific binding, immune serum (I) was used at 1/100 (b) and 1/1000 (c). Non-specific binding was analyzed with preimmune serum at 1/100 (a). (d) The neutralizing monoclonal anti-gp140 antibody TB10 was used as control in the same assay. The previously formed gp140-CD4 complex was labeled with anti-TB10 monoclonal antibodies as fluid ascite diluted at 1/100 (e) and 1/1000 (f), the third graph corresponds to the cell autofluorescence (d).

immunodominant linear antigenic site in this region. However, in contrast to V3 of HIV-1, antibodies produced against linear or cyclic peptide were unable to neutralize SIV infection. This result suggests a different functional role for the V3 region in the SIV replication cycle.

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References

1 Daniel MD, Letvin NL, King NW, Kannagi M, Sehgal PK, Hunt RD, Kanki PJ, Essex M, Desrosiers RC. Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. *Science* 1985; **228**: 1201–1204.
2 Fukasawa M, Miura T, Hasegawa A, Morikawa S, Tsujimoto H, Miki K, Kitamura T, Hayami M. Sequence of simian immunodeficiency virus from African green monkey, a new member of the HIV/SIV group. *Nature* 1988; **333**: 457–461.
3 Kraus G, Werner A, Baier M, Binniger D, Ferdinand FJ, Norley S, Kurth R. Isolation of human immunodeficiency virus-related simian immunodeficiency viruses from African green monkeys. *Proc. Natl. Acad. Sci. U.S.A.* 1989; **86**: 2892–2896.
4 Yu XF, Yu QC, Essex M, Lee TH. The vpx gene of simian immunodeficiency virus facilitates efficient viral replication in fresh lymphocytes and macrophage. *J. Virol.* 1991; **65**: 5088–5091.
5 Javaherian K, Langlois AJ, Schmidt S, Kaufmann M, Cates N, Langedijk JP, Meloen RH, Desrosiers RC, Burns DP, Bolognesi DP, Larosa GJ, Putney SD. The principal neutralization determinant of simian immunodeficiency virus differs from that of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. U.S.A.* 1992; **89**: 1418–1422.
6 Javaherian K, Langlois AJ, Montefiori DC, Kent KA, Ryan KA, Wyman PD, Stott J, Bolognesi DP, Murphey-Corb M, Larosa GJ. Studies of the conformation-dependent neutralizing epitopes of simian immunodeficiency virus envelope protein. *J. Virol.* 1994; **68**: 2624–2631.
7 Benichou S, Legrand R, Nakagawa N, Faure T, Traincard F, Vogt G, Dormont D, Tiollais P, Kieny MP, Madaule P. Identification of a neutralizing domain in the external envelope glycoprotein of simian immunodeficiency virus. *AIDS Res. Hum. Retroviruses* 1992; **8**: 1165–1170.
8 Torres JV, Malley A, Banapour B, Anderson DE, Axthelm MK, Gardner MB, Benjamini E. An epitope on the surface envelope glycoprotein (gp130) of simian immunodeficiency virus (SIVmac) involved in viral neutralization and T cell activation. *AIDS Res. Hum. Retroviruses* 1993; **9**: 423–430.
9 Babas T, Belhadj-Jrad B, Le Grand R, Dormont D, Montagnier L, Bahraoui E. Specificity and neutralizing capacity of three monoclonal antibodies produced against the envelope glycoprotein of simian immunodeficiency virus isolate 251. *Virology* 1995; **211**: 339–344.
10 Steimer KS, Scandella CJ, Skiles PV, Haigwood NL. Neutralization of divergent HIV-1 isolates by conformation-dependent human antibodies to Gp120. *Science* 1991; **254**: 105–108.
11 Moore JP, Sattentau QJ, Wyatt R, Sodroski J. Probing the structure of the human immunodeficiency virus surface glycoprotein gp120 with a panel of monoclonal antibodies. *J. Virol.* 1994; **68**: 469–484.
12 Richardson TM Jr, Stryjewski BL, Broder CC, Hoxie JA, Mascola JR, Earl PL, Doms RW. Humoral response to oligomeric human immunodeficiency virus type 1 envelope protein. *J. Virol.* 1996; **70**: 753–762.
13 Luke W, Coulibaly C, Dittmer U, Voss G, Oesterle R, Makoschey B, Sauermann U, Jurkiewicz E, Stahl-Henning C, Petry H, Hunsmann G. Simian immunodeficiency virus (SIV) gp130 oligomers protect rhesus macaques (Macaca mulatta) against the infection with SIVmac32H grown on T-cells or derived ex vivo. *Virology* 1996; **216**: 444–450.
14 Hoxie JA. Hypothetical assignment of intrachain disulfide bonds for HIV-2 and SIV envelope glycoproteins. *AIDS Res. Hum. Retroviruses* 1991; **7**: 495–499.
15 Chavez LG Jr, Scheraga HA. Intrinsic stabilities of portions of the ribonuclease molecule. *Biochemistry* 1980; **19**: 1005–1012.
16 Furie B, Schechter AN, Sachs DH, Anfinsen CB. An immunological approach to the conformational equilibrium of staphylococcal nuclease. *J. Mol. Biol.* 1975; **92**: 497–506.
17 Sachs DH, Schechter AN, Eastlake A, Anfinsen CB. An immunologic approach to the conformational equilibria of polypeptides. *Proc. Natl. Acad. Sci. U.S.A.* 1972; **69**: 3790–3794.
18 Neurath AR, Strick N, Fields R, Jiang S. Peptides mimicking selected disulfide loops in HIV-1 gp120, other than V3, do not elicit virus-neutralizing antibodies. *AIDS Res. Hum. Retroviruses* 1991; **7**: 657–662.
19 Schulze-Gahmen U, Klenk HD, Beyreuther K. Immunogenicity of loop-structured short synthetic peptides mimicking the antigenic site A of influenza virus hemagglutinin. *Eur. J. Biochem.* 1986; **159**: 283–289.
20 Satterthwait AC, Arrhenius T, Hagopian RA, Zavala F, Nussenzweig V, Lerner RA. Conformational restriction of peptidyl immunogens with covalent replacements for the hydrogen bond. *Vaccine* 1988; **6**: 99–103.
21 Christodoulides M, McGuinness BT, Heckels JE. Immunization with synthetic peptides containing epitopes of the class 1 outer-membrane protein of Neisseria meningitidis: production of bactericidal antibodies on immunization with a cyclic peptide. *J. Gen. Microbiol.* 1993; **139**: 1729–1738.
22 Palker TJ, Matthews TJ, Langlois A, Tanner ME, Martin ME, Scearce RM, Kim JE, Berzofsky JA, Bolognesi DP, Haynes BF. Polyvalent human immunodeficiency virus synthetic immunogen comprised of envelope gp120 T helper cell sites and B cell neutralization epitopes. *J. Immunol.* 1989; **142**: 3612–3619.
23 Wang CY, Looney DJ, Li ML, Walfield AM, Ye J, Hosein B, Tam JP, Wong-Staal F. Long-term high-titer neutralizing activity induced by octameric synthetic HIV-1 antigen. *Science* 1991; **254**: 285–288.
24 Ahlers JD, Dunlop N, Pendleton CD, Newman M, Nara PL, Berzofsky JA. Candidate HIV type 1 multideterminant cluster peptide-P18MN vaccine constructs elicit type 1 helper T cells, cytotoxic T cells, and neutralizing antibody, all using the same adjuvant immunization. *AIDS Res. Hum. Retroviruses* 1996; **12**: 259–272.
25 Kobs-Conrad S, Lee H, DiGeorge AM, Kaumaya PT. Engineered topographic determinants with alpha beta, beta alpha beta, and beta alpha beta alpha topologies show high affinity binding to native protein antigen (lactate dehydrogenase-C4). *J. Biol. Chem.* 1993; **268**: 25285–25295.
26 Merrifield B. Solid phase synthesis. *Science* 1986; **232**: 341–347.
27 Chakrabarti L, Guyader M, Alizon M, Daniel MD, Desrosiers RC, Tiollais P, Sonigo P. Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. *Nature* 1987; **328**: 543–547.
28 Benjouad A, Babas T, Montagnier L, Bahraoui E. N-linked oligosaccharides of simian immunodeficiency virus envelope glycoproteins are dispensable for the interaction with the CD4 receptor. *Biochem. Biophys. Res. Commun.* 1993; **190**: 311–319.
29 Le Grand R, Vogt G, Vaslin B, Roques P, Theodoro F, Aubertin AM, Dormont D. Specific and non-specific immunity and protection of macaques against SIV infection. *Vaccine* 1992; **10**: 873–879.
30 Javaherian K, Langlois AJ, LaRosa GJ, Profy AT, Bolognesi DP, Herlihy WC, Putney SD, Matthews TJ. Broadly neutralizing antibodies elicited by the hypervariable neutralizing determinant of HIV-1. *Science* 1990; **250**: 1590–1593.
31 Burns DP, Desrosiers RC. Selection of genetic variants of simian immunodeficiency virus in persistently infected rhesus monkeys. *J. Virol.* 1991; **65**: 1843–1854.
32 Kanki PJ, McLane MF, King NW Jr, Letvin NL, Hunt RD, Sehgal P, Daniel MD, Desrosiers RC, Essex M. Serologic identification and characterization of a macaque T-lymphotropic retrovirus closely related to HTLV-III. *Science* 1985; **228**: 1199–1201.
33 Letvin NL, Daniel MD, Sehgal PK, Desrosiers RC, Hunt RD, Waldron LM, MacKey JJ, Schmidt DK, Chalifoux LV, King NW. Induction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STLV-III. *Science* 1985; **230**: 71–73.
34 Belhadj Jrad B, Bahraoui E. Antigenicity of linear and cyclic peptides mimicking the disulfide loops in HIV-2 envelope glycoprotein: synthesis, reoxidation and purification. *J. Pept. Res.* 1998; **51**: 370–385.
35 Finnegan CM, Berg W, Lewis GK, DeVico AL. Antigenic properties of the human immunodeficiency virus envelope during cell-cell fusion. *J. Virol.* 2001; **75**: 11096–11105.

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36 Kropelin M, Susal C, Daniel V, Opelz G. Inhibition of HIV-1 rgp120 binding to CD4+ T cells by monoclonal antibodies directed against the gp120 C1 or C4 region. *Immunol. Lett.* 1998; **63**: 19–25.
37 Moore JP, Binley J. HIV: envelope's letters boxed into shape. *Nature* 1998; **393**: 630–631.
38 Moore JP, Nara PL. The role of the V3 loop of gp120 in HIV infection. *Aids* 1991; **5**(Suppl. 2): S21–S33.
39 Moore JP, Trkola A, Dragic T. Co-receptors for HIV-1 entry. *Curr. Opin. Immunol.* 1997; **9**: 551–562.
40 Cormier EG, Dragic T. The crown and stem of the V3 loop play distinct roles in human immunodeficiency virus type 1 envelope glycoprotein interactions with the CCR5 coreceptor. *J. Virol.* 2002; **76**: 8953–8957.
41 Resch W, Hoffman N, Swanstrom R. Improved success of phenotype prediction of the human immunodeficiency virus type 1 from envelope variable loop 3 sequence using neural networks. *Virology* 2001; **288**: 51–62.
42 Rizzuto CD, Wyatt R, Hernandez-Ramos N, Sun Y, Kwong PD, Hendrickson WA, Sodroski J. A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. *Science* 1998; **280**: 1949–1953.
43 Kolchinsky P, Kiprilov E, Bartley P, Rubinstein R, Sodroski J. Loss of a single N-linked glycan allows CD4-independent human immunodeficiency virus type 1 infection by altering the position of the gp120 V1/V2 variable loops. *J. Virol.* 2001; **75**: 3435–3443.
44 Ogert RA, Lee MK, Ross W, Buckler-White A, Martin MA, Cho MW. N-linked glycosylation sites adjacent to and within the V1/V2 and the V3 loops of dualtropic human immunodeficiency virus type 1 isolate DH12 gp120 affect coreceptor usage and cellular tropism. *J. Virol.* 2001; **75**: 5998–6006.
45 Losman B, Bolmstedt A, Schonning K, Bjorndal A, Westin C, Fenyo EM, Olofsson S. Protection of neutralization epitopes in the V3 loop of oligomeric human immunodeficiency virus type 1 glycoprotein 120 by N-linked oligosaccharides in the V1 region. *AIDS Res. Hum. Retroviruses* 2001; **17**: 1067–1076.
46 Fenouillet E, Clerget-Raslain B, Gluckman JC, Guetard D, Montagnier L, Bahraoui E. Role of N-linked glycans in the interaction between the envelope glycoprotein of human immunodeficiency virus and its CD4 cellular receptor. Structural enzymatic analysis. *J. Exp. Med.* 1989; **169**: 807–822.
47 Bahraoui E, Benjouad A, Guetard D, Kolbe H, Gluckman JC, Montagnier L. Study of the interaction of HIV-1 and HIV-2 envelope glycoproteins with the CD4 receptor and role of N-glycans. *AIDS Res. Hum. Retroviruses* 1992; **8**: 565–573.
48 Gruters RA, Neefjes JJ, Tersmette M, de Goede RE, Tulp A, Huisman HG, Miedema F, Ploegh HL. Interference with HIV-induced syncytium formation and viral infectivity by inhibitors of trimming glucosidase. *Nature* 1987; **330**: 74–77.
49 Matthews TJ, Weinhold KJ, Lyerly HK, Langlois AJ, Wigzell H, Bolognesi DP. Interaction between the human T-cell lymphotropic virus type IIIB envelope glycoprotein gp120 and the surface antigen CD4: role of carbohydrate in binding and cell fusion. *Proc. Natl. Acad. Sci. U.S.A.* 1987; **84**: 5424–5428.
50 Robinson WE Jr, Montefiori DC, Mitchell WM. Evidence that mannosyl residues are involved in human immunodeficiency virus type 1 (HIV-1) pathogenesis. *AIDS Res. Hum. Retroviruses* 1987; **3**: 265–282.
51 Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, Beary H, Hayes D, Frankel SS, Birx DL, Lewis MG. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat. Med.* 2000; **6**: 207–210.
52 Roben P, Moore JP, Thali M, Sodroski J, Barbas CF 3rd, Burton DR. Recognition properties of a panel of human recombinant Fab fragments to the CD4 binding site of gp120 that show differing abilities to neutralize human immunodeficiency virus type 1. *J. Virol.* 1994; **68**: 4821–4828.
53 Sanders RW, Venturi M, Schiffner L, Kalyanaraman R, Katinger H, Lloyd KO, Kwong PD, Moore JP. The mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120. *J. Virol.* 2002; **76**: 7293–7305.
54 Reitter JN, Means RE, Desrosiers RC. A role for carbohydrates in immune evasion in AIDS. *Nat. Med.* 1998; **4**: 679–684.
55 Johnson WE, Morgan J, Reitter J, Puffer BA, Czajak S, Doms RW, Desrosiers RC. A replication-competent, neutralization-sensitive variant of simian immunodeficiency virus lacking 100 amino acids of envelope. *J. Virol.* 2002; **76**: 2075–2086.
56 Cole KS, Steckbeck JD, Rowles JL, Desrosiers RC, Montelaro RC. Removal of N-linked glycosylation sites in the V1 region of simian immunodeficiency virus gp120 results in redirection of B-cell responses to V3. *J. Virol.* 2004; **78**: 1525–1539.
57 Benjouad A, Gluckman JC, Montagnier L, Bahraoui E. Specificity of antibodies produced against native or desialylated human immunodeficiency virus type 1 recombinant gp160. *J. Virol.* 1993; **67**: 1693–1697.
58 Zolla-Pazner S, Cohen SS, Krachmarov C, Wang S, Pinter A, Lu S. Focusing the immune response on the V3 loop, a neutralizing epitope of the HIV-1 gp120 envelope. *Virology* 2007.
59 Vogel T, Kurth R, Norley S. The majority of neutralizing Abs in HIV-1-infected patients recognize linear V3 loop sequences. Studies using HIV-1MN multiple antigenic peptides. *J. Immunol.* 1994; **153**: 1895–1904.
60 Bjorling E, Chiodi F, Utter G, Norrby E. Two neutralizing domains in the V3 region in the envelope glycoprotein gp125 of HIV type 2. *J. Immunol.* 1994; **152**: 1952–1959.
61 Robert-Guroff M, Aldrich K, Muldoon R, Stern TL, Bansal GP, Matthews TJ, Markham PD, Gallo RC, Franchini G. Cross-neutralization of human immunodeficiency virus type 1 and 2 and simian immunodeficiency virus isolates. *J. Virol.* 1992; **66**: 3602–3608.
62 Traincard F, Rey-Cuille MA, Huon I, Dartevelle S, Mazie JC, Benichou S. Characterization of monoclonal antibodies to human immunodeficiency virus type 2 envelope glycoproteins. *AIDS Res. Hum. Retroviruses* 1994; **10**: 1659–1667.
63 Babas T, Benichou S, Guetard D, Montagnier L, Bahraoui E. Specificity of antipeptide antibodies produced against V2 and V3 regions of the external envelope of human immunodeficiency virus type 2. *Mol. Immunol.* 1994; **31**: 361–369.
64 Jurkiewicz E, Hunsmann G, Schaffner J, Nisslein T, Luke W, Petry H. Identification of the V1 region as a linear neutralizing epitope of the simian immunodeficiency virus SIVmac envelope glycoprotein. *J. Virol.* 1997; **71**: 9475–9481.
65 Kent KA, Rud E, Corcoran T, Powell C, Thiriart C, Collignon C, Stott EJ. Identification of two neutralizing and 8 non-neutralizing epitopes on simian immunodeficiency virus envelope using monoclonal antibodies. *AIDS Res. Hum. Retroviruses* 1992; **8**: 1147–1151.
66 Kodama T, Burns DP, Silva DP, Veronese FD, Desrosiers RC. Strain-specific neutralizing determinant in the transmembrane protein of simian immunodeficiency virus. *J. Virol.* 1991; **65**: 2010–2018.
67 Palker TJ, Muir AJ, Spragion DE, Staats HF, Langlois A, Montefiori DC. The V3 domain of SIVmac251 gp120 contains a linear neutralizing epitope. *Virology* 1996; **224**: 415–426.

