Recognition Properties of V3-Specific Antibodies to V3 Loop Peptides Derived from HIV-1 gp120 Presented in Multiple Conformations†

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ABSTRACT: To identify structural constraints and amino acid sequences important for antibody recognition of the third variable domain (V3) of HIV-1 gp120, we have studied the solution conformation of three 35-mer circular V3 loop peptides derived from HIV-1 strains which differ in syncytium- (SI) and non-syncytium-inducing (NSI) capacity. In addition to 2D NMR and CD analyses, fluid- and solid-phase immunoassays were performed using V3-specific antibodies to V3 peptides and gp120 derived from different strains of HIV-1. NMR and CD spectroscopy indicated that circular and linear V3 loops exist in water as a dynamic ensemble of multiple conformations. Amino acid substitutions and biochemical modifications of the V3 loop were found to affect antibody binding depending on the presentation of the antigens. From NMR observations and immunological experiments, we provide evidence for a V3 loop specific monoclonal antibody interaction which is directed predominantly against linear epitopes rather than against discontinuous epitopes. The absence of a single defined solution conformation of 35-mer circular V3 peptides should be taken into account when using V3-related peptides to investigate structural elements in the V3 domain of the gp120 envelope protein of HIV-1 involved in biological processes of the virus.

The third variable domain (V3) of the gp120 envelope protein of the human immunodeficiency virus type 1 (HIV-1)[1] has been identified as the principal neutralizing domain of T-cell line adapted viral strains (TCLA) (*1, 2*). The infection of the target cell is made possible by the binding of gp120, found on the surface of the virus and its infected cells, to the CD4 and chemokine receptors CXCR4 or CCR5, protein complexes present on the membrane of the target cell. All HIV-1 strains are able to replicate in peripheral blood lymphocytes, but their capacity to infect and replicate in CD4+ T-cells, macrophages and cell lines, varies substantially. Differences are also observed between the cytopathic effects of different HIV-1 strains. T-cell-tropic strains are able to induce syncytium formation between infected cells (SI), while macrophage-tropic strains are typically of the non-syncytium-inducing (NSI) phenotype (*3, 4*). Cell tropism and the syncytium-inducing capacity of HIV-1 have been ascribed to substitutions of positively charged or neutral amino acids at position 11 and/or 28 in the V3 domain (*5, 6*). Furthermore, fusion between the cell surface and the virus was initially thought to be mediated after proteolytic cleavage at a single site in the V3 loop between Arg^{18} and Ala^{19} by a thrombin-like protease (*7, 8*). Recently, it was found that the V3 loop is involved in binding of gp120 to chemokine receptors; these co-receptors are members of the seven-transmembrane chemokine family (*9*). The primary structure of the V3 domain of a large number of HIV-1 positive individuals was analyzed, and, interestingly, the amino acid region G^{15}-P-G-R(Q)^{18} turned out to be highly conserved among various subtypes (*10*). This part of the V3 loop has been found in earlier studies on isolate-specific peptides to form a β_{II}-turn (*11*−*13*) which is conserved upon interaction with V3-specific neutralizing antibodies. This has been determined by X-ray crystallography of cocrystals and solution NMR analysis (*14*−*17*). Recently, experimental evidence has been provided for an antibody-dependent conformation of the V3 loop (*18*).

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[1] Abbreviations: CD, circular dichroism; DQF-COSY, double quantum filtered correlated spectroscopy; DTT, dithiothreitol; EIA, enzyme immunoassay; gp, glycoprotein; HIV-1, human immunodeficiency virus type 1; Mab, monoclonal antibody; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; NSI, non-syncytium inducing; Pab, polyclonal antibody; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RCM, reduction−carboxymethylated; RIA, radioimmunoassay; SI, syncytium inducing; TCLA, T-cell line adapted virus; TFE, 2,2,2-trifluoroethanol; TOCSY, total correlation spectroscopy.

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Phenotype	peptide	Amino acid position 1 11 28 35
NSI-consensus		CTRPNNNTRK **S** I-HI--GPGRAFYTTG **E** IIGDIRQAHC
a- p160B-al-IHI	(circular 35-mer V3)	A **Q**
b- p160B-al-IHM	(circular 35-mer V3)	M A **Q**
SI-consensus MN		CTRPNYNKRK **R** I-HI--GPGRAFYTTK **N** IIGTIRQAHC
c- ACH-Q17	(circular 35-mer V3)	N I G **Q** N
d- SP155-MN tip-V3	(15-mer short V3)	K **R** I-HI--GPGRAFYTTK
SI-IIIB (LAI)		CTRPNNNTRK **S** I-RI**QR**GPGRAFVTIGK **I** -G NMRQAHC
e- SP104-IIIB tip-V3	(15-mer short V3)	I-RI**QR**GPGRAFVTIG

FIGURE 1: Amino acid sequences of the circular V3 loop peptides of HIV-1, which have been used for NMR, CD analyses, and immunological experiments (a, b, and c). A consensus sequence for NSI and SI variants, respectively, is also listed (*10*), as well as short 'tip of the loop' peptides (d and e) which have been used primarily for generating mouse Mabs MN-V3-53 and IIIB-V3-13 (*31*), and second for the application in immunological assays. The amino acid residues at positions 11 and 28, determining the SI/NSI phenotype of HIV-1, are indicated in boldface type (*5, 6*).

The structural elements within the V3 loop that induce both strain-specific neutralizing antibodies and involvement in biological phenotype of the virus were analyzed using 2D NMR and CD analysis. We therefore set out to determine the solution conformation of different circular consensus sequences of the V3 region of both syncytium-inducing (SI) and non-syncytium-inducing (NSI) HIV-1 isolates (*3*−*6*). The reactivity of these V3 peptide antigens with cognate antibodies was also studied.

Presented here is a 2D NMR and CD study of the conformation of three cyclic 35-mer V3 loop peptides in water and in aqueous solutions containing TFE: cV3-Q17, derived from a syncytium-inducing HIV-1 isolate and two cV3 peptides; p160B-al, derived from a nonsyncytium−macrophagetropic HIV-1 isolate. The last two cyclic peptides differ only in one amino acid residue, in which I^{14} (isoleucine) is replaced by M^{14} (methionine). Alignment of the three peptides with consensus sequences for SI- and NSI-HIV-1 strains is shown in Figure 1.

Complex formation between cV3 peptides and variants thereof with in vivo and in vitro generated V3-specific antibodies was studied in solid- and fluid-phase assays. We introduced amino acid substitutions, linearization, and cleavage of the loop to investigate the influence on V3 peptide−antibody interaction. Purified recombinant gp120 envelope protein of HIV-1 was also used in the study to investigate the effects of modification of the third variable domain.

MATERIALS AND METHODS

Peptides. The peptides used in this work consisted of three cyclic V3 loops and two short peptides to test the effect of amino acid substitutions on the antibody reactivity (Figure 1). The peptides SP104 and SP155 were prepared as described (*19*). Purity was checked by analytical HPLC. The circular V3 peptides were purchased from Zeneca (Cambridge Research Biochemicals, England) and were of greater than 95% purity. NMR experiments were carried out on the cyclic and linear (only IHI) 35-mer V3 loop peptides (Figure 1, a−c). The composition and sequence were checked by one-dimensional NMR spectroscopy. No NMR experiments were carried out on the short V3 peptides SP104 and SP155 (Figure 1, d,e).

Preparation of Peptides for NMR and CD Spectroscopy. Samples for NMR spectroscopy were prepared by dissolving lyophilized peptides in 90% ^1H_2O and 10% ^2H_2O to a final concentration of about 1 mM (20 mM phosphate buffer, pH 5). Dithiothreitol (DTT) was added to the sample of the linear form of V3-160B-al-IHI to ensure that no intermolecular disulfide bonds were formed. The 20% (v/v) trifluoroethanol/water solutions were obtained by adding the appropriate amount of deuterated trifluoroethanol to the water sample. For the exchange measurements, 10 mg of cV3p160B-al-IHI was dissolved in 20 mM phosphate buffer, pH 7 (10% deuterated). Solubility of the peptides was high in all cases, the only exception being the linear p160 IHI dissolved in TFE/water.

NMR Spectroscopy. NMR experiments were performed on Bruker DRX600 and VARIAN Unity Inova 500 spectrometers equipped with digital phase shifting hardware. All experiments were carried out at 5 °C. Both pH and temperature values were chosen to minimize amide exchange (*20*). Two-dimensional double quantum filtered correlated spectroscopy (DQF-COSY) (*21*), nuclear Overhauser enhancement spectroscopy (NOESY) (*22*), and total correlation spectroscopy (TOCSY) (*23, 24*) data were acquired according to standard procedures (*25*). Mixing times of 150 and 250 ms were used in the NOESY experiments. Suppression of the H_2O peaks was achieved by means of semiselective pulse sequences based on the jump−return sequence (*26*, *27*) in combination with weak continuous irradiation of the residual water resonance. The program NMRPipe (*28*) was used for transformation of all data. The time domain was zero-filled to the next power of 2 and multiplied by phase-shifted sine-bells. All spectra were analyzed using the program XEASY (*29*).

Circular Dichroism Spectroscopy. The CD spectra were recorded on a Jasco J-715 spectrometer in the 260−190 nm range. The peptide concentration used was 20 μM (20 mM phosphate buffer, pH 7). Spectra were recorded at 5 °C. The TFE/water solutions were obtained by adding the appropriate amount of TFE to the water sample. Spectra were usually accumulated 8 times. Mean residue ellipticities were calculated for each sample by the usual method (*30*).

HIV-1 Glycoprotein gp120. The NIH AIDS Research and Reference Program provided the recombinant gp120 protein from HIV-1 strain MN. The recombinant gp120 from strain IIIB was expressed via a baculovirus expression system in

insect cells, and was kindly provided by PHAGE, La Jolla, CA.

Antibodies. The mouse monoclonal antibody IIIB-V3-13, produced by one of us (J.D.L., ref *31*), was generated against SP104, a V3-loop-IIIB-related peptide (cf. Figure 1). The antibody provided by NIH AIDS Research and Reference Reagent Program (nr: 1727) can neutralize TCLA-HIV-1-IIIB (SI) in vitro. The mouse monoclonal antibody MN-V3-53 has not been described before. Briefly, a group of three mice received injections of V3 peptide SP155 conjugated to BSA mixed with Specol (a water-in-oil adjuvant) subcutaneously on the upper side of both hind feet. Antibody responses were monitored weekly with a lectin- and V3-peptide-EIA (see below) on serum samples. After 4 weeks, mice received a booster injection with the same dose via the same route. A mouse with high response was selected for fusion and received additional injections. Four days after this second booster, the spleen was used for fusion. Fusion was performed according to a standard protocol (*32*). Limiting dilution was performed for subcloning of positive clones. Ascites fluid was produced in BalB/c mice. Mouse immunoglobulins were isolated by affinity chromatography on protein A−Sepharose beads according to the instructions of the manufacturer (Pharmacia, Uppsala, Sweden).

Antibody MN-V3-53 recognized homologous V3 peptides (cf. Figure 1). Mab-MN-V3-53 can neutralize TCLA-HIV-1 strain MN in vitro. The antibody did not bind appreciably to an unrelated but also highly glycosylated protein like C1-inhibitor or to unrelated peptides. The antibody will be described in more detail elsewhere.

DO142.10 is a recombinant human Fab fragment (Fd + light chain) and was initially rescued from a Phage Display Library (*33*−*35*). Drs. D. R. Burton and P. W. I. Parren from the Scripps Research Institute, La Jolla, CA, kindly provided the M13 phage containing the Fab fragment. Fab-DO142.10 can neutralize TCLA-HIV-1 strain MN in vitro and also binds to MN-like V3 peptides (*35*). Rabbit polyclonal antibodies 50112 were prepared against cV3 peptide p160B-al-IHI (cf. Figure 1). Briefly, a rabbit received injections of 20 μg of cV3-p160B-al peptide mixed with Freund's adjuvants subcutaneously at four different places. Antibody responses were monitored with a V3 peptide EIA on serum samples. After 2 weeks, the rabbit received a booster injection with the same dose via the same route. This was repeated 4 times. The serum taken after the final booster was used for the experiments described here.

Serum Samples. From a large panel of HIV-positive serum samples, we selected specimens representing different stages of HIV-1 infection. From individual HIV+/W, we obtained sequential serum samples at the time of seroconversion (*36*). MGT is an HIV-1 positive individual without clinical symptoms and seroconverted after infection with an HIV-1 NSI phenotype at least 10 years ago. The serum contains moderate to high titers of neutralizing antibodies. The chimpanzee Maya was experimentally infected with HIV-1 strain IIIB (*37*). The serum sample used in this study was taken 7 years after infection and was kindly provided by Dr. John Heeny from the Biomedical Primate Research Centre BPRC, Rijswijk, The Netherlands.

Thrombin Digestion. Thrombin (EC 3.4.21.5) from human plasma was purchased from Sigma Chemical Co. (St. Louis, MO). Hirudin from leeches was also obtained from Sigma. Thrombin digestion of peptides and gp120 was performed according to Clements et al. (*7*). After completion of cleavage, checked by SDS−PAGE, thrombin activity was inhibited by the addition of an excess of hirudin and the protease inhibitors PepStatin and PMSF (both from Sigma Chemical Co.).

Biochemical Modification of V3 Peptides and gp120 Proteins. To study the effect of chaotropic agents on recognition by antibodies, purified V3 peptides and gp120 were treated with urea and urea/DTT at final concentrations of 4 M and 4 M/25 mM, respectively. After immobilization to the plates, the antigens were washed extensively to remove chaotropic agents.

Reduction−Carboxymethylation of V3 Peptides and gp120 Proteins. The antigens were dissolved in guanidine hydrochloride and dithiothreitol at final concentrations of 0.8 M and 30 mM, respectively. After 1 h incubation on ice, iodoacetamide was added to a final concentration of 85 mM. The mixture was allowed to incubate for 1 h at 4 °C in the dark. The reduced and alkylated V3 peptides (only circular V3 peptides were used) and gp120 proteins were dialyzed against PBS, aliquoted, and stored at −20 °C.

^{125}I-Radiolabeling of V3 Peptides, gp120 Proteins, and Antibody. In brief, 50 μL solutions of PBS containing either 20 μg of peptides or 5 μg of protein were used for labeling with Na ^{125}I using chloramine T for 30 s (*38*, *39*). The radiolabeled preparations were purified from free iodine by dialysis, and subsequently aliquoted in the presence of protease inhibitor PMSF and bovine serum albumin (fraction V, Sigma) in a final concentration of 0.1% and stored until use at −20 °C.

gp120 EIA. We used a gp120 capture enzyme immunosorbent assay (EIA) for mapping studies and studying the effect of reduction−carboxymethylation and for the study of thrombin cleavage on the reactivity of antibodies with gp120. Briefly (*40*), a lectin, *Galanthus nivalis* agglutinin (Boehringer Mannheim GmbH, Germany), was used to coat plastic Falcon 3911-96 U-bottom well plates (Becton Dickinson Labware, Franklin Lakes, NJ) at a concentration of 0.5 μg/well in PBS. After coating for 16 h at room temperature, the plates were washed 2 times with demineralized water and subsequently blocked for 30 min at 37 °C with Tris-buffered saline containing 4% (w/w) nonfat milk powder (Campina, Eindhoven, The Netherlands) and 0.5% gelatin (Merck, Darmstadt, Germany). The plates were washed with Tris-buffered saline containing 0.01% Tween-20. Native and treated recombinant gp120 were captured to the plate via preadsorbed lectin. The antigens and antibodies were used at saturating concentrations or, if this was not possible, at the highest concentration that would allow completion of the experiments. In comparative studies of antibody binding in solid- and fluid-phase assays, the antibodies were applied in serial dilutions. Purified antibodies were generally used between 1 and 15 μg/mL and diluted in Tris-HCl (pH 7.5)-buffered saline containing 0.4% nonfat milk powder, 0.1% gelatin, and 0.1% TX-100 or NP-40 (Sigma Chemical Co.). The antibodies were allowed to react with gp120 during a 2 h incubation at 37 °C. The unbound reagents were removed by washing with Tris-buffered saline containing 0.01% Tween-20. Goat antibodies conjugated with alkaline phosphatase and specific for either mouse, rabbit, or human IgG, or for human kappa light chain, were used to monitor

the binding of antibodies. These conjugates were purchased from Pierce (Rockford, IL). Phosphatase substrate tablets were obtained from Sigma Chemical Co. Color development was followed at 405 nm. Chimpanzee antibody binding was detected by rabbit antibodies specific for human IgG conjugated with horseradish peroxidase (CLB Sanquin Blood Supply, Department of Immune Reagents, Amsterdam, The Netherlands). Color development was initiated by addition of the substrate tetramethylbenzidine (Merck, Darmstadt, Germany) and hydrogen peroxide, and followed at 450/540 nm.

Peptide EIA was performed as follows: V3-related peptides, RCM-V3-peptides, peptides in the presence of chaotropic reagents (see before), and thrombin-cleaved peptides were solubilized in PBS at 10 μg/mL. The peptides were immobilized on Falcon plates (see before) during an overnight incubation at room temperature. The EIA was performed as described above for gp120.

Binding of gp120 and V3 Peptides to Immobilized Antibody (RIA Format). The binding studies of antibodies to V3 peptides and gp120 proteins were performed as follows: an optimal amount of antibodies or serial dilutions of antibodies or sera were mixed with an excess of protein A−Sepharose beads suspended in PBS containing 0.1% bovine serum albumin and 0.05% NP-40. After 2 h of incubation head-over-tail, the beads were washed 4 times with phosphate-buffered saline (PBS) containing 0.01% Tween-20. Antibodies bound to protein A−Sepharose beads were incubated head-over-tail with radiolabeled gp120 or V3 peptide (approximately 100 000 cpm) in PBS containing 0.1% bovine serum albumin and 0.05% NP-40, for 16 h at room temperature. After washing the beads with PBS/0.01% Tween-20, bound radioactivity was measured in a gamma counter.

RESULTS AND DISCUSSION

Structural Properties of Circular and Linear V3 Loop Peptides (35-mers) Derived from HIV-1 with Distinct Phenotypes (SI and NSI): NMR

Resonance Assignments for the Peptides. The proton resonances were assigned according to established strategies (*20*) using DQF-COSY and TOCSY spectra to recognize amino acid spin systems and NOESY spectra to identify sequential connectivities. Low chemical shift dispersion resulted in extensive overlap of the resonances in both aqueous and TFE/water solutions. Nevertheless, an almost complete assignment could be achieved for all backbone and side chain proton resonances (Supporting Information). No assignment could be made for the linear p160IHI solved in 20% TFE/water, due to aggregation of the sample. Figure 2 gives a representative example of all observed sequential NOE connectivities [$d_{\alpha N}(i,i+1)$, $d_{\beta N}(i,i+1)$, and $d_{NN}(i,i+1)$] (all data are available as Supporting Information). Sequential NOE connectivities of the α-protons of Arg3 and Gly15 with the δ-protons of Pro4 and Pro16, respectively, evidence that in all peptides both Xaa−Pro amide bonds have the trans configuration in the major isomer. Signals attributable to a *cis*-proline residue were also observed in the spectra of these peptides. The assignment of the signals of this minor isomer was not possible due to its low population and the strong overlap of its signals with those of the major all-trans isomer. Henceforth, all structural considerations refer to the all-trans isomer.

NMR Studies on the V3 Peptides in Water: A Dynamic Mixture of Multiple Conformations. By means of 2D NMR and CD spectroscopy, we studied the solution conformation of the V3 loops derived from primary isolates of HIV-1: the T-cell-tropic Q17 (SI), the macrophage-tropic p160-B-al NSI (IHM), and the circular and linear forms of p160-B-al (IHI).

A number of our observations demonstrate that these V3 loops exist in aqueous solution as a dynamic ensemble of multiple conformations. The backbone and side chain chemical shifts closely approach typical random coil values (*41*) (with a spread of $\Delta\delta < 0.2$ ppm). Also, the Wishart−Sykes chemical shift indexes (*42*) predict no regular secondary structure on the basis of the observed α-proton chemical shifts. The observed $^3J_{N\alpha}$ couplings range between 6.3 and 7.8 Hz, except for the alanine residues, again indicating conformational averaging (*41*).

The observed NOE connectivities also confirm the disordered state of the peptides. Diagnostic of a helical conformation is the presence of a stretch of NOE connectivities between the amide protons of consecutive residues $d_{NN}(i,i+1)$, supported by the presence of medium-range $d_{\alpha\beta}(i,i+3)$, $d_{\alpha N}(i,i+3)$, and $d_{\alpha N}(i,i+4)$ connectivities. The diagnostic NMR evidence for a β-turn consists of one or more $d_{NN}(i,i+1)$ NOEs within the turn, accompanied by a reduction in the magnitude of the corresponding $d_{\alpha N}(i,i+1)$ NOEs. A $d_{\alpha N}(i,i+2)$ connectivity across the turn is a requirement for a positive identification of the turn. Long-range $d_{\alpha\alpha}(i,j)$ and $d_{\alpha N}(i,j)$ NOEs are diagnostic of β-sheet structures. In Figure 2, a scheme is presented illustrating the NOE connectivities observed for each cV3 peptide. Although a number of the $d_{NN}(i,i+1)$ NOEs are seen in the NOESY spectra of the NSI- and SI-derived V3 peptides, there is no evidence for $d_{\alpha\beta}(i,i+3)$, $d_{\alpha N}(i,i+3)$, or $d_{\alpha N}(i,i+4)$ NOEs, which rules out the presence of an ordered α-helix. No evidence for a defined β-structure is seen either. Despite the presence of strong $d_{\alpha N}(i,i+1)$ NOEs, the NOESY spectra do not show the expected interstrand NOEs characteristic of β-sheet formation.

In short, the consistent presence of both strong $d_{\alpha N}(i,i+1)$ and relatively strong $d_{NN}(i,i+1)$ NOEs indicates that all peptides sample a broad range of conformations, irrespective of the striking amino acid sequence variations between SI- and NSI-derived V3 loop peptides (*5*, cf. Figure 1). They do not have a single, specific, conformation.

In concordance with these observations, the CD spectra (Figure 3) of the four peptides in aqueous solution are typical of random-coil peptides, with a negative extremum at 196 nm.

The $d_{NN}(i,i+1)$ NOE connectivities, mainly observed in the C-terminal region of the peptides, could be indicative of nascent helix in solution (*42*). Further support for the presence of such a helix in the form of a number of $d_{\alpha N}(i,i+2)$ NOEs (*43*) is, however, not apparent from the NMR spectra. The nascent helix itself consists of a population of different conformations, in which a significant proportion contains backbone conformations in the α-region of (ϕ,ψ) space in the Ramachandran plot (*44*), rather than of any single defined solution conformation.

Previous NMR studies on the complete V3 loop of the MN (*11*, *45*) [Chang Mai (*13*), RF (*46*), and NSI-macroph-

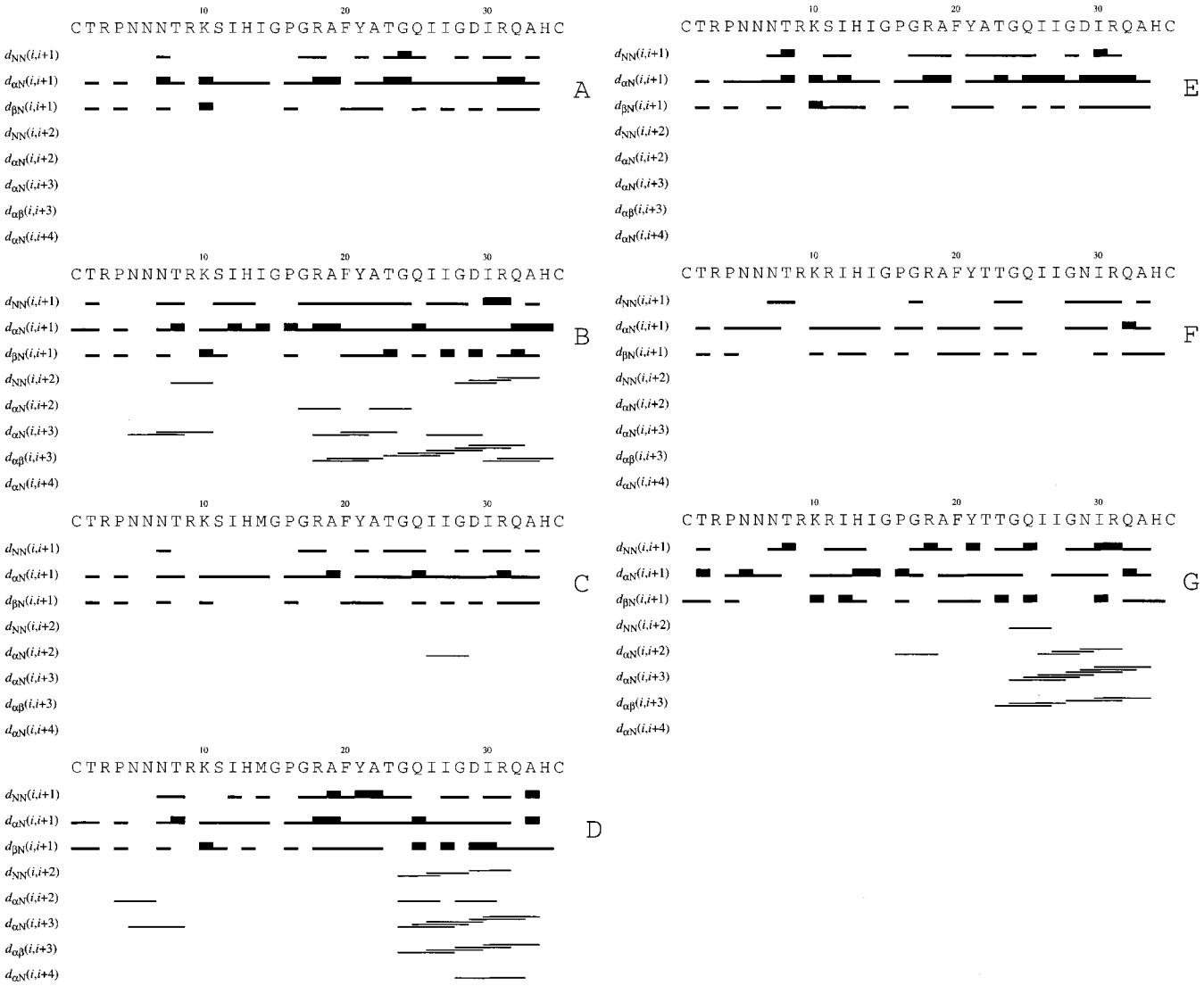

FIGURE 2: Schematic diagrams showing the magnitude of various NOE connectivities (small, thin lines; medium, thick lines; strong, filled boxes) observed in the NOESY spectra of the cyclic V3 peptides in water and 20% TFE/water solutions. Schematic diagrams of (A) cyclic p160B-al (IHI) in water, (B) cyclic p160B-al (IHI) in 20% TFE/water, (C) cyclic p160B-al (IHM) in water, (D) cyclic p160B-al (IHM) in 20% TFE/water, (E) linear p160B-al (IHI) in water, (F) cyclic Q17 in water, and (G) cyclic Q17 in 20% TFE/water.

age-tropic consensus (*47*)] indicated the presence of a β-turn in the conserved GPGR region. This conclusion was based on the presence of a $d_{NN}(i,i+1)$ NOE between residues 17 and 18 and secondary structure prediction (*13, 45*) and on a combination of the above-mentioned $d_{NN}(i,i+1)$ NOE and a diagnostic NOE connectivity that establishes the presence of a turn conformation which is the $d_{\alpha N}(i,i+2)$ NOE across the turn between the Pro16 and the Arg18 (*11, 46, 47*).

We do see a $d_{NN}(i,i+1)$ NOE between the amide protons of Gly17 and Arg18, which is evidence for a population of conformers with backbone conformations in the β-turn region of (ϕ,ψ) space. However, this peak is also typical for a population of conformers in the α-region. For the cV3-Q17 peptide, we could not observe the diagnostic NOE between residues 16 and 18. For all the other peptides, a very small peak is present, but it could not unambiguously be assigned to this connectivity due to overlap of resonances. Although we cannot totally exclude the possibility of a β-turn in this region, the NOE is small, and all other data do not support this conclusion. We therefore conclude that the peptides have multiple conformations in aqueous solution. A similar result

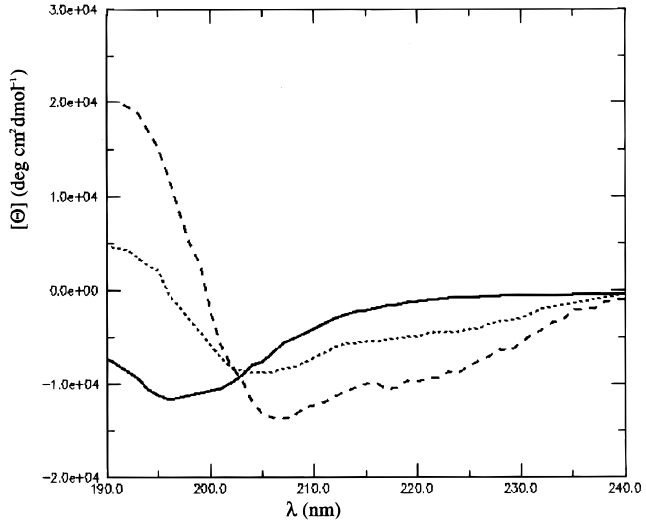

FIGURE 3: CD spectra of cyclic V3p160B-al (IHI) in water (solid line), in 20% TFE/water solution (dotted line), and in 50% TFE/water solution (dashed line).

was obtained by solid-state NMR measurements on a small peptide comprising the 24 central residues in the V3 loop of strain IIIB. These measurements showed a heterogeneous population of peptide conformations in the unbound form, whereas an antibody-dependent conformation is adopted in the bound state (*18*).

Furthermore, an 18 residue HIV-1IIIB V3 peptide in complex with the Fv fragment of an anti-gp120 antibody reveals an unexpected type VI β-turn comprising residues of the tip. The central glycine and proline of this turn are linked by a cis peptide bond (*48*). This confirms the hypothesis of an antibody-dependent conformation of the bound V3 loops.

NMR Studies on the cV3 Peptide in TFE/Water Solution: A Tendency for α*-Helical Structure in the C-Terminus.* In a 20% TFE solution, the C-terminal part of the analyzed peptides is predominantly helical. A group of $d_{\alpha N}(i,i+2)$, $d_{NN}(i,i+2)$, $d_{\alpha N}(i,i+3)$, and $d_{\alpha\beta}(i,i+3)$ NOEs (cf. Figure 2) is observed for the sequence Gly17−His34 in the NOESY spectra of three peptides, showing clearly the helical structures in this region of the peptides. The low intensity of these NOEs indicates that the peptides are still highly flexible. Small upfield shifts of 0.01−0.15 ppm are observed for all α-proton resonances in the sequence Thr23−Arg31 [except for Gly28 in Q17, Asp29 in cV3-p160(IHM), and Gly28, Asp29 in cV3-p160(IHI)]. Since intrinsic changes in the chemical shifts, of α-protons as a function of trifluoroethanol concentration in random-coil peptides, are negligible (*41*), these upfield shifts are consistent with a tendency to form a α-helical structure in this region of the molecule upon trifluoroethanol addition. $^3J_{N\alpha}$ couplings were difficult to measure, probably due to a combination of smaller coupling constants (α-helical structure) and broader lines. A tendency to form α-helical structure could also be observed by the analysis of the CD spectra of the peptides in 20% and 50% TFE solutions. A representative example is given Figure 3. Addition of TFE apparently mediates formation of helical structures although this amounts to maximally 25−30% of the total peptide concentration (calculated from the accepted value of −36 000 to −40 000 deg cm^2 dmol^{-1} at 222 nm for 100% helix). The addition of TFE results in an isodichroic point in the CD spectra (Figure 3), a two-state equilibrium.

Finally, the presence of the NOE connectivity $d_{\alpha N}(i,i+2)$ between the α-proton of the Pro16 and the N-proton of the Arg18, which indicates the presence of a turn conformation, was vaguely manifested in the NOESY spectrum of the peptide Q17 only.

Although the V3 peptides have a tendency to form a better defined structure in the presence of TFE, no structural differences are seen between the peptides derived from different phenotype HIV-1 isolates, as regards cell tropism and the cytopathic effect.

In summary, the NMR studies demonstrate that under physiological conditions circular V3 peptides do not form a well-defined structure, but that they consist of a dynamic ensemble of multiple conformations. No structural differences are seen between the V3 peptides derived from different phenotypes of HIV-1, neither in aqueous nor in water/TFE solutions. To further explore these observations, a systematic evaluation of the binding of V3-specific antibodies to the third variable domain of gp120 and V3 specific peptides was performed. We applied several series of circular, and shorter linear V3 peptides and gp120 proteins (see Figure 1) derived from different HIV-1 phenotypes, in fluid- and solid-phase immunoassays.

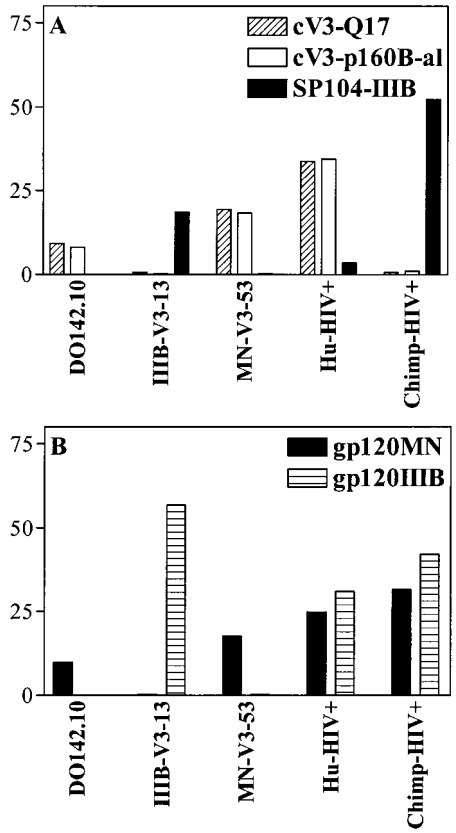

FIGURE 4: V3-specific monoclonal antibodies were applied in a radioimmunoassay to analyze their recognition pattern with homologous and heterologous V3 peptides and gp120 derived from different strains of HIV-1, e.g., HIV-1 IIIB versus Q17/p160B-al/MN strains. The radiolabeled antigens were presented in a fluid-phase assay. As positive controls, a serum sample from an HIV+ individual MGT (Hu-HIV+) and a serum sample from a chimpanzee experimentally infected with HIV-1 IIIB (Chimp-HIV+) were used. The binding of the antibody is expressed in percent bound radiolabeled antigens calculated from the input value. Values are corrected for nonspecific binding.

Structural Properties of V3 Loop Peptides and Their Interaction with V3-Specific Antibodies: Immunology

Detection of Amino Acid Sequence Based Interactions between V3-Specific Antibodies and V3 Peptides and gp120: EIA and RIA. To further study the solution conformation of circular V3 loop peptides, we compared the binding of three V3-specific monoclonal antibodies to homologous and heterologous V3 peptides and gp120 presented in a fluid-phase assay (RIA, Figure 4) and solid-phase assay (EIA, Figure 5). We used mouse Mab IIIB-V3-13, raised against IIIB-V3-SP104 peptide, mouse Mab MN-V3-53, raised against MN-V3-SP155 peptide, and a recombinant human Fab fragment, DO142.10, elicited in vivo against HIV-1. We also used a serum sample from an HIV-1-infected individual (MGT) and a serum sample from a chimpanzee experimentally infected with HIV-1-IIIB. We studied the effect of amino acid sequence variations in the V3 loop and the third variable domain of gp120 on the binding of V3 antibodies and serum samples. Figures 4 and 5 show that the Mabs and serum samples display sequence-

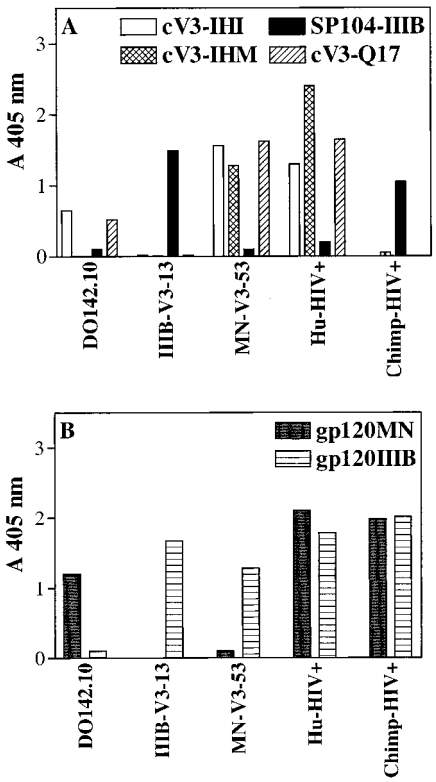

FIGURE 5: V3-specific monoclonal antibodies were applied in an enzyme immunoassay to analyze their recognition pattern with homologous and heterologous V3 peptides and gp120 derived from different strains of HIV-1, e.g., HIV-1 IIIB versus Q17/p160B-al-(IHI or IHM)/MN strains. The peptide and protein antigens were presented to the antibodies from a solid phase. The same positive control sera were used as described in Figure 4 (Hu-HIV+ and Chimp-HIV+). The antibody binding is corrected for nonspecific binding of negative control antibodies and conjugates to the immobilized antigens.

determined specificity for both V3 peptides and gp120. On one hand, Mab IIIB-V3-13 and chimpanzee serum react exclusively with IIIB-peptides/gp120, while on the other hand Mab MN-V3-53, Fab DO142.10, and HIV+ MGT bind to p160B-al- and Q17/MN-related antigens only (cf. Figure 1). These differences are most likely related to the insertion of amino acids Q-R before the tip residues, GPGR, in the IIIB peptide, with the most profound difference with the p160B-al, MN, and Q17 sequences, although we cannot exclude that amino acid variations in the N- or C-terminal part of the peptides may contribute to the absence of binding (cf. *18*, *31*). Strikingly, despite the presence of a charged amino acid (R) at position 11 in SP155, the immunogen for Mab MN-V3-53, the antibody did bind to p160B-al-IHI (NSI) sequences. The HIV+ serum MGT (anti-NSI) and Fab D0142.10 (anti-NSI) also bound to both SI- and NSI-related V3 peptides. This finding is consistent with the absence of phenotype-determined solution conformations of cV3 loop peptides. The absence of cross-reactivity in a fluid-phase assay of the used Mab panel is not surprising since the V3 loops of both circular V3 peptides used in our study are conformationally heterogeneous in the unbound form and differ significantly in primary amino acid sequences compared with IIIB. Nonetheless, an example has been reported of a Mab (RP142-59.1) raised against the MN variant of V3 which also binds to IIIB V3 loop *(49*, *50*). Cross-reactivity was established by EIA and neutralization assays with HIV-1 TCLA strains MN and IIIB, respectively. An unexpected observation was that the neutralization capacity of RP142-59.1 for the homologous strain MN was low to moderate but high for the heterologous strain HIV-1 IIIB. Most anti-V3 Mabs directed against one HIV strain are completely unreactive even with closely related strains, due to amino acid variations in the hypervariable immunogenic tip of the loop (*51*). However, Welicky and co-workers (*18*) provided evidence to explain the binding of RP142-59.1 to the GPGR motif (neutralization domain) of gp120-IIIB. In solid-state NMR studies, the GPGR motif of IIIB was found to be conformationally heterogeneous in the unbound state, and it appeared to have sufficient conformational flexibility to allow binding of antibody RP142-59.1 raised against a V3-MN peptide. This raises the question as to whether neutralizing (GPGR-binding) antibodies raised against V3-IIIB might exist that would also bind to V3-MN variants of gp120. The large immunogenic differences between the IIIB-type V3 loop and the variants lacking the Q-R insertion are further underscored by our finding that none of several rabbit polyclonal antibodies raised against a variety of MN, Q17, and p160B-al V3 peptides bound V3-IIIB peptides in fluid-phase binding assays (J.D.L. and J.G.H., manuscript in preparation).

The importance of primary amino acid sequence for recognition by antibodies against V3 loops is also shown by the finding that Fab DO142.10 does not bind to a peptide in which Ile^{14} in p160B-al (IHI) has been substituted with a methionine residue (Figure 5). From the EIA experiments (Figure 5), we suggest that serum sample MGT and monoclonal antibodies display some cross-reactivity with heterologous peptide SP104-IIIB which might be an assay format dependent phenomenon (see also below).

Detection of Amino Acid Sequence Based Interactions between V3-Specific Antibodies and V3 Peptides and gp120 in RIA: Effects of Reduction−Carboxymethylation and Cleavage by Thrombin. The effect of reduction−carboxymethylation of circular V3 peptides was analyzed for Mab MN-V3-53 and serum MGT; a circular V3 peptide of IIIB was not available. Figure 6 shows that the linearization of cV3p160B-al (IHI) resulted in a lower antibody binding consistent with (partial) loss of epitopes as a result of the treatment. However, NMR analysis had previously shown that reduced peptides tend to aggregate (see above) which might also explain the decrease in binding of the antibodies. Thrombin cleavage of cV3p160B-al, RCM-V3p160B-al, and gp120 from HIV-1 strain MN resulted in a complete loss of binding of Mab MN-V3-53 and Fab DO142.10 (the latter is not shown). Similarly, thrombin cleavage of IIIB peptide SP104 and HIV-1 gp120 strain IIIB totally abolished binding of Mab IIIB-V3-13. For the thrombin-cleaved peptides, similar results were observed for serum samples HIV+ MGT and HIV-1-IIIB chimpanzee (the latter is not shown). The sensitivity of the third variable domain of gp120 and V3 peptides for the cleavage by thrombin confirms our NMR analysis that a type II β-turn centered at Pro^{16} and G^{17} is absent in our V3 peptides. As published by Johnson et al. (*52*), a conformational rearrangement of the V3 loop for docking thrombin is most likely centered at the conserved residue Pro^{16}. For antibodies in MGT and chimpanzee serum, the cleavage of gp120 by thrombin resulted in a partial decrease in binding. The effect of thrombin cleavage on the epitopes of the V3 loop is obscured by the reactivity of this

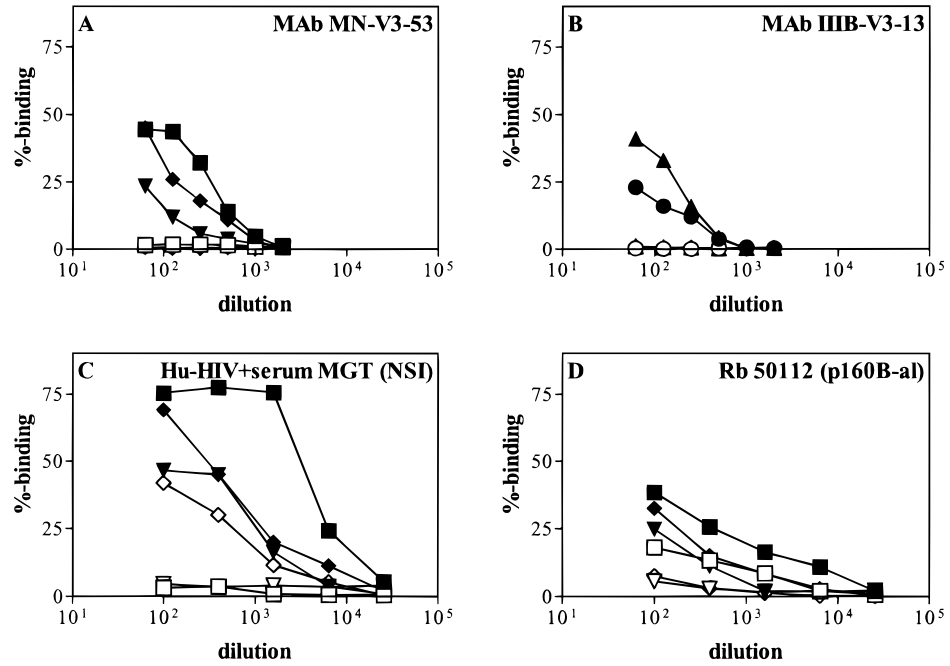

FIGURE 6: V3 loop peptides and gp120 derived from HIV-1 strains IIIB, MN, and p160B-al were used in a fluid-phase assay (RIA), before (closed symbols) and after (open symbols) treatment with thrombin. The binding of serial dilutions of Mabs MN-V3-53 and IIIB-V3-13, HIV+ human serum sample MGT (the sample contained HIV-1 isolates with NSI phenotype), and the positive control rabbit polyclonal antibody 50112 (immunized with cV3-p160B-al) to homologous and heterologous peptides was compared compared (■ = cV3 p160B-al IHI, ▼ = reduction−carboxymethylated cV3 p160B-al IHI, ● = sp104 IIIB, ◆ = gp120MN, and ▲ = gp120IIIB).

serum with other epitopes on gp120. The absence of reactivity of HIV-positive serum samples with thrombin-cleaved peptides and gp120 in a fluid-phase assay is remarkable considering that these sera contain polyclonal antibodies against HIV-1 envelope antigens. The absence of reactivity of serum MGT and Mab MN-V3-53 with cleaved cV3 could not be ascribed to degradation of the peptide since the presence of V3-antigenic determinants could still be demonstrated for rabbit serum 50112 containing antibodies raised against the cV3p160Ba-l peptide (Figure 6). It remains to be determined whether reduced binding of antibodies to thrombin-cleaved peptides or gp120 protein occurs because the cleavage site is part of the epitope or whether destruction of conformers is responsible for loss of epitopes. To investigate this, thrombin-digested V3 loop peptides were presented on solid matrixes and tested with serial dilutions of the antibodies. As apparent from Figure 7, Mabs IIIB-V3-13, MN-V3-53, and HIV+ serum sample [MGT, Chimpanzee serum, and a HIV+ serum sample (W) taken 12 weeks after seroconversion] still bind, although at reduced levels, to thrombin-cleaved peptides in the EIA format. Thus, in contrast to the findings with the fluid-phase format, at least part of the coated−cleaved peptides still contains the epitopes of both mouse Mabs and HIV+ sera. The reduced binding is not the result of improper binding of cleaved antigens to microtiter wells, since rabbit serum 50112 binds to both cV3p160Ba-l and cleaved cV3 at the same level. The difference between the two types of assays may be due to fixation of the solid-phase-bound peptides in a structure favorable for recognition by the antibodies. Moreover, the local concentration of immobilized peptides is higher than the concentration of peptide in solution, thus increasing the binding of low-affinity antibodies.

Binding studies similar to the ones described above were performed with sequential serum samples from an HIV+ individual (W) taken at seroconversion and thereafter (see also Figure 7). We compared the antibody binding to circular V3 peptides before and after cleavage with thrombin in a solid-phase (EIA format) and fluid-phase assay (RIA format). The results are depicted in Figure 8. The antibody responses to both intact and thrombin-cleaved circular V3 peptides increased over time in the solid-phase assay, but again responses were negative for thrombin-cleaved V3 peptide if presented in the fluid-phase assay. The inability of HIV+ sera to bind to thrombin-digested V3 peptide, and the decreased binding to thrombin-cleaved gp120 as found in a fluid-phase assay, was also confirmed in a larger panel (n = 25) of HIV-1 positive serum samples obtained from individuals at different stages of the disease (data not shown). A host-cell-mediated V3 loop proteolysis in the viral life cycle has not been demonstrated unequivocally (*7, 8, 52*), but a role for immunophilins has been suggested to induce a conformational cis to trans isomerization of Pro^{16}, a prerequisite for cleavage by a thrombin-like protease (*53*). We speculate that the inability of HIV+ sera to bind to proteolytically cleaved V3 loop may result in an impairment of the humoral response in controlling putative V3-mediated steps in the entry process of the virus.

Detection of Amino Acid Sequence Based Interaction between V3-Specific Antibodies and V3 Peptides and gp120: Effects of Treatment with Chaotropic Agents and Reduction−Carboxymethylation (EIA). To further investigate structural constraints for antibody binding, V3 peptides and gp120 proteins were either treated with chaotropic agents or subjected to reduction followed by carboxymethylation. These experiments showed that binding of Mabs to their homologous V3 peptides was not affected by either of the treatments (data not shown). This suggests that binding of the Mabs to V3 peptides is not sensitive to denaturation, although we cannot rule out the possibility that the structure

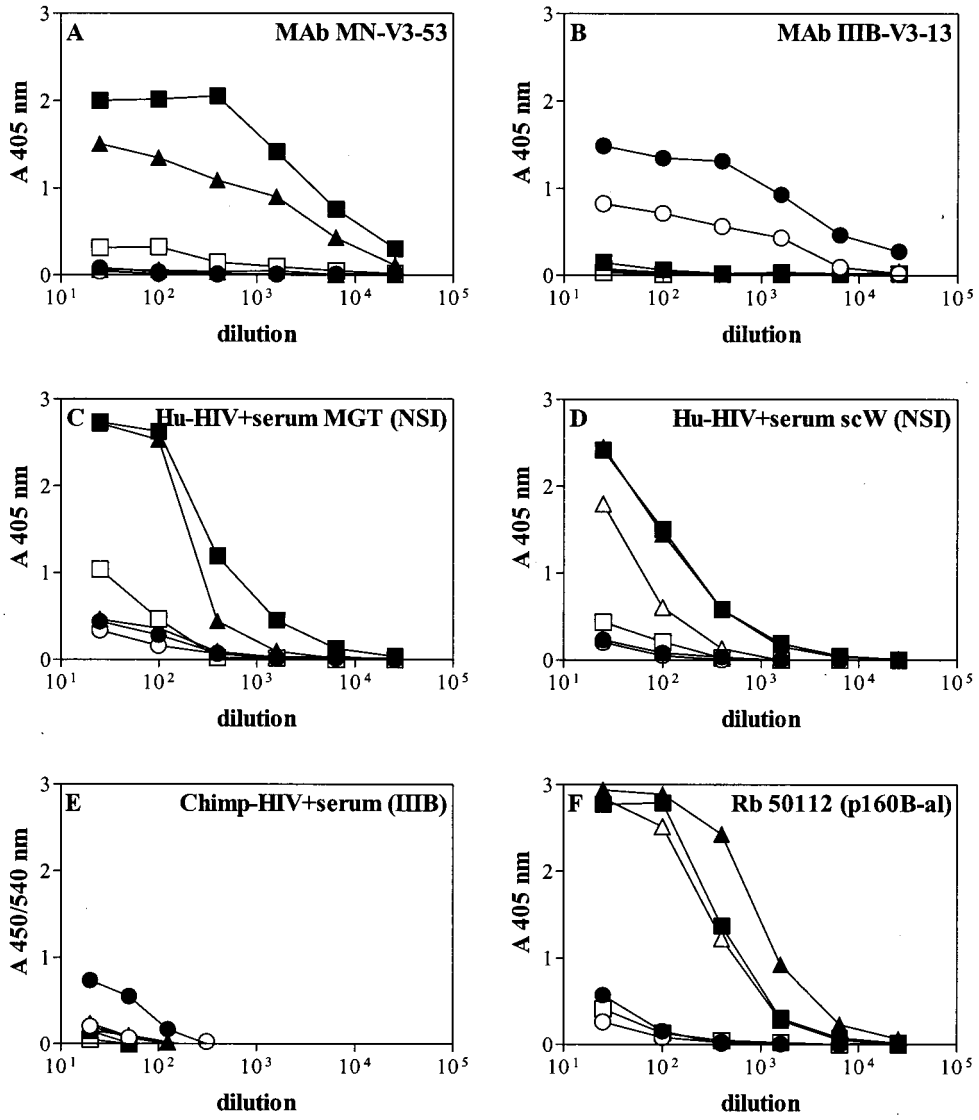

FIGURE 7: V3 loop peptides and gp120 derived from HIV-1 strains IIIB, MN, and p160B-al were used in a solid-phase assay (EIA), before (closed symbols) and after (open symbols) treatment with thrombin. The binding of serial dilutions of Mabs MN-V3-53 and IIIB-V3-13, HIV+ human serum samples MGT and scW (both samples contained HIV-1 isolates with NSI phenotype), HIV+ chimpanzee serum sample from a chimpanzee experimentally infected with HIV-1 IIIB (Chimp-HIV+), and the positive control rabbit polyclonal antibody 50112 (immunized with cV3-p160B-al) to homologous and heterologous peptides was compared (■ = sp155 MN, ▲ = cV3 p160B-al IHI, and ● = sp104 IIIB).

of the peptides, if any, had been restored after removal of the chaotropic agents. However, together with the data from NMR studies, these results suggest that linear epitopes, which could exhibit internal flexibility, rather than discontinuous epitopes, in the peptides determine the binding site for the antibodies. In a discontinuous epitope, the amino acids involved are not necessarily arranged in a primary sequence. In addition, the structure of a discontinuous epitope is sensitive for biochemical modifications, e.g., denaturing and proteolytic cleaving of the antigen. Figure 9 depicts the results of studies on the reactivity of the V3-specific Mabs and two HIV+ sera with RCM-gp120. Again, strain-specific binding is demonstrated for IIIB-V3-13, MN-V3-53, and Fab DO142.10. As with the peptides, the binding of Mab IIIB-V3-13 is resistant to reduction−carboxymethylation of gp120-IIIB. The binding of MN-V3-53 to MN gp120 was only partly affected by reduction−carboxymethylation of the protein. These findings are in agreement with the assumption that peptide-induced antibody responses are mainly directed against linear epitopes and as a consequence antibody reactivity is predominantly amino acid sequence determined. For Fab DO142.10, elicited in vivo against HIV-1, treatment of gp120 by reduction and carboxymethylation (RCM) has a negative effect on antibody binding. This indicates that medium- and long-range interactions in the folded gp120 protein codetermined the binding site in the third variable domain of gp120. This is in contrast with the observations obtained with peptides. Thus, V3 peptide molecules used in our study only partly reflect the epitopes as present on HIV-1 gp120.

CONCLUSIONS

The experiments described in this paper show that the V3 peptides of several strains form a heterogeneous ensemble of conformations in solution. The NOE data, the *J*-coupling values, and the chemical shifts, in combination with CD measurements, show that the peptides, regardless of their NSI or SI phenotype, are a dynamic mixture of conformers, of which a very small fraction forms an α-helix at the C-terminus and a *β*-turn in the GPGR region. These

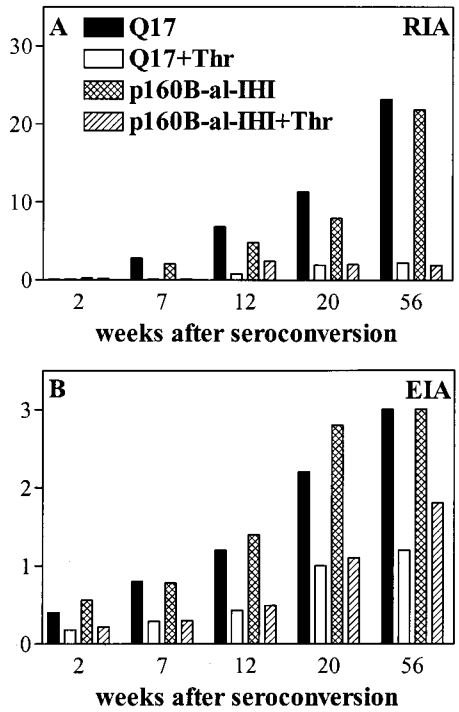

FIGURE 8: Comparison of the recognition pattern of sequential serum samples from an HIV+ individual (scW) taken shortly after seroconversion (2 weeks) with a year follow-up to cV3 peptides Q17 and p160B-al (IHI), presented in a solid-phase assay (EIA) and ^{125}I-radiolabeled peptides in a fluid-phase assay (RIA). TheV3-related peptides were presented as intact peptides as well as after treatment with thrombin. The values are not corrected for nonspecific binding. A serum sample taken before seroconversion (*36*) shows a background of 1.2% for RIA and an OD at $A_{405\ nm}$ of 0.050 for EIA.

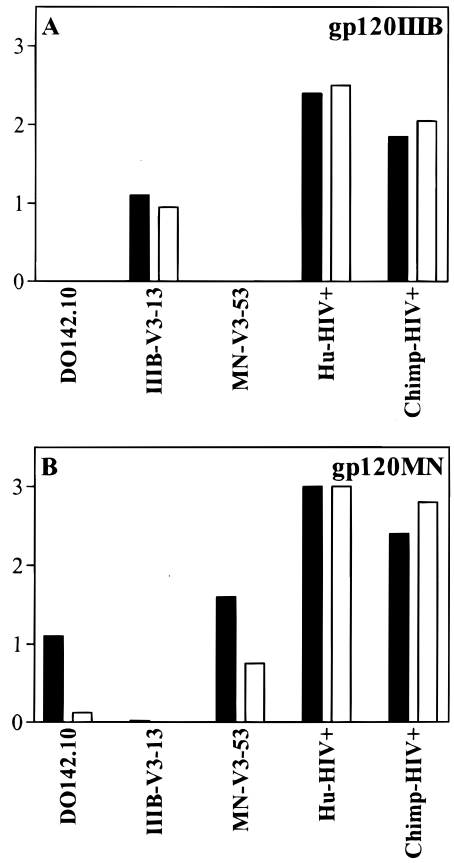

FIGURE 9: Analysis of three site-specific V3 antibodies in their binding pattern to homologous and heterologous HIV-1 gp120 derived from strains IIIB and MN. The gp120 proteins are presented on a lectin-coated EIA plate before (native, solid bars) and after biochemical modification of the proteins by reduction−carboxymethylation (open bars). The same positive control sera were used as described in Figure 4 (Hu-HIV+ and Chimp-HIV+).

observations are in agreement with the conclusion of Welicky et al. (*18*), that the GPGR motif of the V3 loop is conformationally heterogeneous in the unbound form. Despite the fact that the peptides derived from different strains of HIV-1 all lack defined structures in solution, the peptides presented in the fluid-phase assay are still differentially recognized by a number of antibodies. This shows that the specificity of the antibody is directed against linear epitopes rather than directed against discontinuous epitopes in V3 peptides. The context of the GPGR motif of Q17/MN/p160B-al differs significantly from the (Q-R)GPGR motif of HIV-1 IIIB (*18*, *31*, *48*), and these differences are probably responsible for the absence of binding of heterologous antibodies. Upon cleavage with thrombin, the mixture of conformations in solution becomes even more complex and abolishes antibody recognition, even for a large number of polyclonal antibodies containing serum samples from HIV-1-infected individuals. We speculate that the lack of antibody recognition for the cleaved third variable domain of gp120 could have consequences for controlling an HIV-1 entry. In a solid-phase assay, however, cleaved V3 peptides still contain sufficient antigenic structure to interact with V3-specific Mabs and sera. This suggests assay-dependent antibody binding. This can be brought about by assuming a fixation of the peptides in a solid-phase assay in a structure favorable for recognition by the antibodies. Moreover, the local concentration of immobilized peptides is higher than the concentration of peptides in solution, thus increasing the binding of low-affinity antibodies. From biochemical modification studies of the gp120 protein, we conclude that in solid-phase assays the recognition of the V3 loop by antibodies is dependent on the presence of a linear epitope but that some structures in the folded gp120 protein codetermined the binding site in the third variable domain of gp120.

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SUPPORTING INFORMATION AVAILABLE

Tables with ^{1}H resonance assignments of the following peptides: cV3-p160B-al (IHI) in water solution, cV3-p160B-al (IHI) in 20% TFE solution, cV3-p160B-al (IHM) in water solution, cV3-p160B-al (IHM) in 20% TFE solution, linearized V3-p160B-al (IHI) in water solution, cV3-Q17 in water solution, cV3-Q17 in 20% TFE solution (8 pages). This

material is available free of charge via the Internet at http://pubs.acs.org.

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