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Antibody recognition of synthetic peptides mimicking immunodominant regions of HIV-1 p24 and p17 proteins

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SUMMARY

The *gag* gene of HIV-1 encodes a single open reading frame of 55 kDa that contains three subdomains: the matrix domain (p17), the capsid domain (p24) and the nucleocapsid domain (p15). The p24 and p17 proteins have a predominant α-helical structure and perform important functions throughout the viral life-cycle. The determination of *gag*-specific antibodies is important because declining titers of these antibodies herald clinical deterioration. In this work we present the results obtained on immunoreactiviy of synthetic peptides that mimic immunogenic a-helical regions of p24 and p17. The influence on the immunoreactivity of structural modifications in native sequences, including the addition of non immunogenic side chains: AAAC- and -CAAA on both side of minimal epitopes was evaluated in indirect and competitive enzymeimmunoassays. The conformational characteristcs to the peptides were analysed by circular dichroism and these results were correlated with that obtained in the immunoassays. It was shown that the reactivity of peptides mimicking short α-helical regions of p24 and p17 is improved by adding short non immunogenic chains on both N- and C- terminus. These modifications enhanced the immobilization of the peptides onto the solid support and allowed more accesibility to the minimal epitopes by specific antibodies, in solution.

Key words: HIV-1, capsid protein p24, matrix protein p17, dissociation constants, secondary structure, synthetic epitopes.

RESUMEN

Reconocimiento por anticuerpos de péptidos sintéticos que imitan regiones inmunodominantes de las proteínas p24 y p17 de VIH-1. El gen *gag* del VIH-1 codifica una región de 55kDA que contiene tres subdominios: matriz (p17), cápside (p24) y nucleocápside (p15). Las proteínas p24 y p17 tienen una estructura predominante helicoidal y cumplen un rol importante en el ciclo de vida del virus. En este trabajo presentamos los resultados de inmunorreactividad de péptidos sintéticos que imitan regiones helicoidales de p24 y p17. Utilizando enzimoinmunoensayos se evaluó la influencia de modificaciones en las secuencias nativas sobre la capacidad de reconocimiento de anticuerpos específicos en solución y en fase sólida, incluyendo el agregado de cadenas no inmunogénicas en ambos extremos de los epitopes mínimos. La conformación de los péptidos se determinó por dicroísmo circular y los resultados se correlacionaron con los de inmunorreactividad. Se observó que la capacidad de reconocimiento de anticuerpos por péptidos pequeños que imitan estructuras helicoidales de p24 y p17 mejoró con el agregado de cadenas no inmunogénicas en ambos extremos de los epitopes. Estas modificaciones mejoran la inmovilización sobre las superficies sólidas y permiten una mayor accesibilidad de los anticuerpos a los epitopes mínimos en solución.

Palabras clave: VIH-1, p24, p17, constantes de disociación, estructura secundaria, epitopes sintéticos

INTRODUCTION

The *gag* gene of Human Immunodeficiency virus type 1 (HIV-1) encodes a single open reading frame of 55 kDa. The $Pr55^{Gag}$ is made up of three distinct subdomains, the matrix domain (p17), the capsid domain (p24) and the nucleocapsid domain (p15), each of which is released from the precursor by the action of the virion protease (10).

Capsid protein p24 from HIV-1 has a predominant a-helical structure as was previously determined by circular dichroism (CD) studies (6). The carboxyl-terminal domain, residues 146 to 231, is required for capsid dimerization and viral assembly (11). This domain contains a stretch of 20 residues, called the major homology region (MHR), which is conserved across retroviruses and is essential for viral assembly, maturation, and infectivity. The crystal structure revealed that the globular domain is composed of four helices and an extended amino-terminal strand (11).

Matrix protein p17 from HIV-1 forms an icosahedral shell associated with the inner membrane of the mature virus. Genetic analyses have indicated that the protein performs important functions throughout the viral life-cycle, including anchoring the transmembrane envelope protein

on the surface of the virus, assisting in viral penetration, transporting the proviral integration complex across the nuclear envelope, and localizing the assembling virion to the cell membrane (4).

The three-dimensional solution- and solid-state structures of the (HIV-1) matrix protein have been determined by NMR and X-ray crystallographic methods (2). The first 104 aa of the matrix protein form a single globular domain composed of five helices and capped by a three-stranded mixed beta-sheet.

The matrix protein exists as a monomer in solution at low millimolar protein concentrations, but forms trimers in three different crystal lattices. A relevant conformational change occurs during virion assembly and disassembly (2, 20).

Synthetic peptides corresponding to a single continuous epitope increase the specificity of an immunoassay. When used in solid phase assays, the peptides can be adsorbed directly to the plastic of microtiter plates or can be used as peptide carrier conjugates. In solution peptides can be used as inhibitors of the reaction between antibodies and antigens. Because the antigenic activity of synthetic peptides can vary greatly in different immunoassay formats, it is important to test if the peptide is able to recognize antibodies in different assay formats (21).

Whether the entire epitope or a part of it is simulated by the peptides, there is still the problem of shape. Many short peptides are disordered in solution, while the corresponding part of the protein is under the constraints of the tertiary structure of the folded protein (14, 29).

In this report we present the results obtained on immunoreactivity of peptides that mimic a-helical regions of p24 and p17 HIV-1 proteins containing previously identified epitopes. The different behavior of the peptides while adsorbed on the microtiter plate as well as free in solution was determined by means of indirect and competitive ELISA.

The influence on the immunoreactivity of structural modifications in native sequences, including the addition of non immunogenic side chains: AAAC- and -CAAA on both side of the epitopes (N- and C- terminal part respectively) was evaluated. We have found that the modifications improved the reactivity of peptides containing short α-helical regions. The conformational characteristics of the native and modified sequences were analysed by circular dichroim and these results were correlated with that obtained in the immunoassays.

MATERIALS AND METHODS

Peptide synthesis

Peptides were manually synthesized as C-terminal carboxamide by the solid phase method with the 9-fluorenyl-methoxycarbonyl (Fmoc) strategy (1, 18). Rink amide resin [4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl) phenoxy-resin)] (NovaBiochen, USA) was used to prepare the amide peptides.

The peptides were purified by solid phase extraction using C18 columns (Bakerbond, J.T.Baker, USA) and by preparative reversed phase liquid chromatography (HPLC- Gilson, France) using a C18 column (Vydac, USA), and water/ acetonitrile linear gradients. The purity of the peptides was determinated by analytical HPLC (Gilson) on a reversed phase column (C18, Beckman).

Cyclic peptide versions were prepared by careful oxidation of Cys residues with I_2 solution, using peptide concentrations of 0.1mgr/ml in amonium bicarbonate buffer 0.03 M, pH 7.9. In these conditions it was possible to minimize unwanted dimerization and oligomerization. The reaction was monitored by the Ellman colorimetric assay, and at the same time by HPLC.

Enzyme immunoassays

Test samples

Serum samples were collected from blood of uninfected and infected HIV-1 patients. Serum samples were previously evaluated by commercial ELISA, and the HIV-1 positives were confirmed by Western Blot. The samples were stored at –20 °C.

Fourteen negative and twenty four positive serum samples were used in this study. Positive samples were obtained from patients in differents stages of infection showing p17 and p24 bands in Western Blot. Positive and negative samples were used to prepare positive and negative pools respectively.

Indirect ELISA

The peptides were adsorbed onto polystyrene plates (COSTAR EIA Microplates, cat n° 2580) using 100 µl of a solution containing 10 mg of peptide in 100 ml of carbonate/bicarbonate buffer (pH 9.6). Microplates were incubated 30 min at 37 °C with 100 µl/well of human serum followed by incubation with peroxidase-conjugated anti-human IgG (Sigma) 30 minutes at 37 °C. The plates were developed by adding a solution containing 3,3',5,5'-Tetramethylbenzidine (TMB) and H_2O_2 as substrate. After 30 minutes, the reaction was stopped by adding H_2SO_4 and read at 450 nm (15).

Specificity and sensitivity.

The cutoff value was defined as the mean absorbance of non-reactive sera plus two standard deviations.

The sensitivity and specificity were calculated as follows: (30)

$$\text{Sensitivity} = \frac{\text{reactive sera (ELISA)}}{\text{true positive sera (WB)}} \times 100$$

$$\text{Sensitivity} = \frac{\text{non - reactive sera (ELISA)}}{\text{true negative sera}} \times 100$$

Competition ELISA assays. Determination of dissociation constants (Kd).

In order to compare the ability of the synthetic peptides to bind to specific antibodies in solution, antigen-antibody dissociation constants (K_d) were determined according to the Friguet method (9) and the corrections described by Stevens (28) and Seligman (26).

Antibody titers of positive sera were determined by indirect assays. A dilution into the linear portion of this curve was selected for each peptide to perform competition assays.

Peptides at concentration ranges from 10^{-3}M up to 10^{-9} M, were incubated for 30 min at 37 °C with 100 µl of diluted sera. This solution was used for the detection of the remaining specific antibodies by an indirect ELISA

Considering these results, the fraction of bound antibodies (θ) was determined according to Friguet et al (9):

$$\theta = \frac{A_o - A_i}{A_o - A_{bl}}$$

were A_0 is absorbance in the absence of competing peptide, A_i is absorbance in presence of competing peptide and A_{bl} is the absorbance of the blanck. By using the Steven's correction for bivalent antibodies (28), θ was substituted by *f,* were *f* is $(\theta)^{1/2}$.

Plotting *f* % (inhibition percent) vs molar concentration of competing peptide, the inhibition peptide- antibodies curves were obtained.

Dissociation Constants (K_d) are estimated as the slope of the line obtained by plotting a_0/f versus 1/(1-f), according to Seligman's equation:

$$a_0/f = Kd / (1-f) + i_0$$

were a_0 is the initial concentration of the competing peptide, and i_0 is the initial antibody concentration.

Circular dichroism measurements

CD spectra were recorded with a Jasco J-720 spectropolarimeter over the wavelength range of 195-240 nm using quartz cuvettes of 1-mm path length. Measurements were made on peptide sample concentrations within a range of 0.15-0.8 mg/ml, in deionized water (Mili Q) or 10mM Glycine plus 150 mM NaCl pH2.5, at 20 °C. Some experiments were done in the presence of a structure-promoting solvent such as trifluoroethanol (31).

Secondary structure estimation

Analyses of the secondary structure were performed using the Self Consistent Method (SELCON) developed by Sreerama and Woody (27). The CD spectra were obtained in mdegree ellipticity (q) scale. They were transformed to molar ellipticity [q] using the residue mean weight and concentration prior to the secondary structure analysis.

RESULTS

Peptides corresponding to three different immunogenic regions of p24 core protein were synthesized: p24-1n (aa 196-224), p24-2n (aa 287-307) and p24-3n (aa 303-326) and another one from the N-terminus of p17 matrix protein, named as p17-1n (aa 8-25). These sequences contain continuous epitopes previously characterized (3, 13, 16, 22, 24). The peptide sequences used in this work correspond to the viral strain HXB2 described in HIV Sequence Compendium (HIV Sequence Database, Los Alamos National Laboratory, Los Alamos, USA, available in HIV/SIV SEQUENCE LOCATOR (http://www.hiv.lanl.gov/content/hiv-db/LOCATE_SEQ/locate.html).

Modified native peptide sequences corresponding to the mentioned regions of p24 and p17 were also prepared. In this case non immunogenic chains (AAAC y CAAA) were added on both N and C terminus respectively. These peptides were identified as p24-1ml, p24-2ml, p24-3ml and p17-1ml. Both linear (ml) and cyclic (mc) versions of all these peptides were prepared. Synthetic peptide sequences are shown in Table 1.

Indirect ELISA

In a preliminary study, the reactivity of all the peptides was tested by means of ELISA using pools of positive and negative serum samples. All the peptides except p24-2n, p24-3n and p17-1n were able to distinguish between both pools.

Peptides p24-1ml, p24-1mc, p24-2n, p24-2ml and p17-1n showed 100% of specificity when evaluated in ELISA using a panel of fourteen negative serum samples. The specificity of peptides p24-3n and p24-3mc was 92.86% and for p24-1n, p24-2mc, p24-3ml, p17-1ml and p17-1mc was 85.71%.

Twenty four HIV-1 Western Blot-positive serum samples were selected for screening the immunoreactivity of p24 and p17 peptides.

For p24-1 peptides, seventeen sera showed reactivity to p24-1n (71%), fourteen to p24-1ml (58%) and eleven to p24-1mc (46%). For p24-2 peptides, five sera showed reactivity to p24-2n (21%), thirteen to p24-2ml (54%) and twelve to p24-2mc (50%). For p24-3 peptides, three sera showed reactivity to p24-3n (12.5%), thirteen to p24-3ml (54%) and six to p24-3mc (25%).

Table 1. Synthetic peptide sequences of HIV-1 p24 and p17 proteins

Peptide identification	Sequence
p24-1n (196-224)	AAMQMLKETINE**EAAEWDRVH**PVHAGPIA
p24-1ml	*AAAC*TINE**EAAEWDRVHP**VHAG*CAAA*
p24-2 (287-307)	QGPKEP**FRDYVDRFYK**TLRAE
p24-2ml	*AAAC*GPKEP**FRDYVDRFYK**TLRAE*CAAA*
p24-3n (303-326)	TLRAE**QASQEVKNWMTETL**LVQNA
p24-3ml	*AAAC*TLRAE**QASQEVKNWMTETLL**VQNA*CAAA*
p17-1n (18-25)	LSGG**ELDRWEKIRLR**PGG
p17-1ml	*AAAC* LSGG**ELDRWEKIRLR**PGG*CAAA*

The mininal epitopes are in bold letters. The non-native side chains are in italic letters. The cyclic modified versions (mc) were obtained by oxidation the non-native Cys residue in the lineal modified (ml) peptide.
The peptide sequences used in this work correspond to the viral strain HXB2 described in HIV Sequence Compendium (HIV Sequence Database, Los Alamos National Laboratory, Los Alamos, USA, available in HIV/SIV SEQUENCE LOCATOR (http://www.hiv.lanl.gov/content/hiv-db/LOCATE_SEQ/locate.html).

The highest immunoreactivity was observed with p24-1n, p24-2ml and p24-3ml. None of p24 peptides were able to recognize the totality of the positive serum samples tested (n=24). In most cases it was observed that p24 modified cyclic peptides were less immunoreactives than the linear ones.

For p17 peptides, three positive sera were reactive to p17-1n (12.5%), eighteen to p17-1ml (75%) and eleven to p17-1mc (46%). As it was observed with p24-2 and p24-3 peptides, the modified linear sequence p17-1ml showed the highest immunoreactivity.

According to these results, p24-1n was considered the most immunoreactive of the twelve tested peptides corresponding to *gag* proteins. Additional immunochemical evaluations were done with p24-1n using an extended panel of 69 positive and 41 negative serum samples; a sensitivity of 90.9% and specificity of 95.12 % was found for this antigen (16).

Competitive ELISA: determination of dissociation constants (Kd).

In order to compare the reactivities in solution of the three peptides containing the same epitope (p24-1, p24-2, p24-3 and p17-1, native, and modified versions), inhibition tests of a pool of reactive serum samples were carried out. In this way it was possible to calculate the dissociation constants for the different peptide-antibody complexes.

The selected positive serum samples were mixed in equal parts and the pool was titrated in an indirect assay; a dilution in the medium part of the titration curve was chosen.

Peptides showing the highest immunoreactivity in indirect ELISA (p24-1n, p24-2ml, p24-3ml and p17-1ml) were used as immobilized antigens in competitive assays. It was established that 10^{-5} to 10^{-7} M is the optimal range of peptide concentrations for the inhibition assays, within which the Kd were calculated. In all cases, the remaining antibodies were measured by indirect ELISA.

The inhibition curves and the calculated K_d for p24 and p17 peptides are shown in Figure 1 and Table 2 respectively.

Peptide p24-1n has more affinity for specific antibodies than the modified (linear and cyclic) p24-1 peptides (Figure 1a). According to Figure 1b, p24-2ml has the greatest ability to inhibit specific antibodies in solution (lowest Kd) of the three p24-2 peptides.

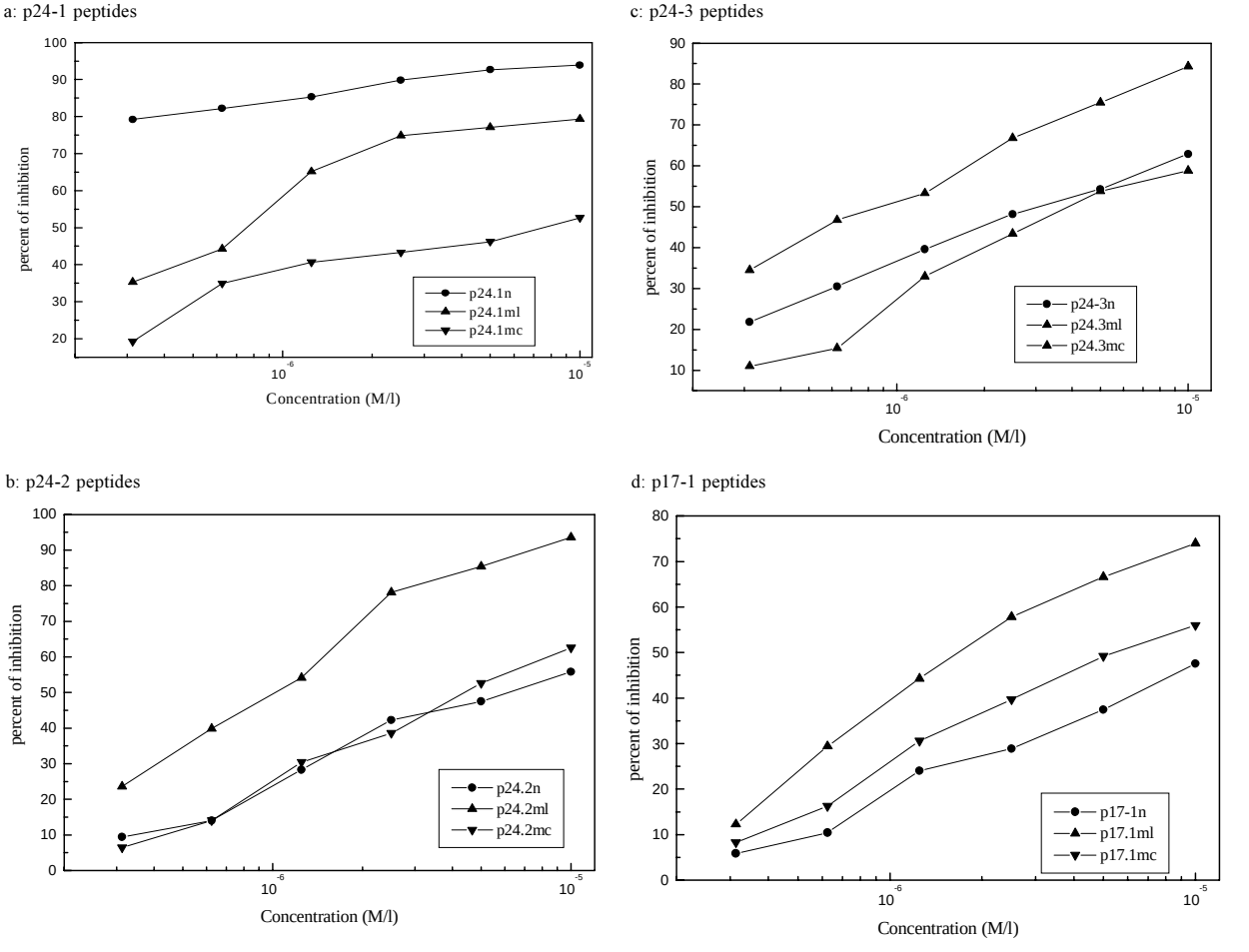

Figure 1

The same was established for p24-3ml and p17-1ml, (Figures 1c, and 1d). These results suggest that, for peptides p24-2, p24-3 and p17-1, the modified linear forms are the most reactive. These results agree with those observed in the indirect ELISA.

CD spectroscopy and estimated secondary structure content

The CD spectrum of peptide p24-1n in water was characterized by two minima about 202 and 222 nm, and suggested a periodic conformation. A maximum at 195 nm and two minima near 207 and 223 nm which are characteristics of a-helix structure, were observed in the presence of trifluoroethanol (TFE). In these conditions 42% a-helix content was calculated for this peptide by the SELCON program. The CD spectra of p24-1ml (linear form) and p24-1mc (cyclic disulfide) were characterized by minima near 201nm (31).

Peptide p24-2n was characterized by one minimun at 201 nm; p24-2ml and p24-2mc by two minima near 206 and 223 nm and similar shapes in this interval. In the presence of 50 and 80% TFE, the CD spectra of p24-2ml showed a maximun at 195 nm and two minima at about 208 and 222 nm; a 40% a-helix content was calculated by the SELCON program for this peptide.

The CD spectra of p24-3 and p17 have similar shapes, and showed one minimun close to 200 nm. In all cases the amplitude of the minimum signals were greater in the linear (p24-3ml and p17ml) than in the native and modified cyclic forms. The different percentages of secondary structure calculated by SELCON are shown in Table 3.

Table 2. Kd values of p24 and p17 peptides

Peptide	Kd native	Kd lineal modified	Kd ciclic modified
p24-1	3,9057 10^{-7} M	1,2384 10^{-6} M	6,9607 10^{-6} M
p24-2	4,4789 10^{-6} M	3,4369 10^{-7} M	3,2655 10^{-6}M
p24-3	3,3946 10^{-6} M	1,0759 10^{-6} M	3,756 10^{-6} M
p17-1	6,892 10^{-6} M	1,8778 10^{-6} M	4,4681 10^{-6} M

DISCUSSION

HIV-1 infection induces antibody responses to the viral structural proteins encoded by the genes *env* (gp120 and gp41) and *gag* (p24 and p17), but as the disease progress to AIDS the level of antibodies to the HIV-1*gag* proteins falls. Consequently the reduction in antibody reactivity to HIV-1 p24 and p17 is an early predictor of disease progression (5, 6, 10, 11).

The selection and utilization of highly sensible and specific synthetic antigens from p24 and p17 HIV-1 proteins for antibody recognition assays may contribute to perform longitudinal analyses of the immune response in infected patients during the course of the infection.

The first 104 aa of the matrix protein p17 form a single globular domain composed of five helices and capped by a three-stranded mixed beta-sheet (2). Peptide p17-1 sequence is close to the N-terminus of p17 and contains a minimal size hydrophilic epitope (ELDRWEKIRL,

Table 3. Percentages of secondary structures for p24 and p17 peptides calculated by SELCON 2

Peptide	% α-helix	% β total	Turns	unordered	% total	%error
P24-1n	13	32	27	26	99	0.17
P24-1ml	7	39	26	27	98	0.18
P24-1mc	8	37	27	28	99	0.13
p24-1n + 80% TFE	42	13	23	21	99	0.14
P24-2 n	3	39	23.7	32.3	98	0.13
P24-2ml	17.8	41	24.5	16.8	100.1	0.16
p24-2mc	15	32.2	21.5	31	99.7	0.14
p24-2ml + 50%TFE	39.49	27.54	20.72	11.36	99.11	0.09
p24-2ml + 80%TFE	39.93	27.89	20.20	12.23	100.25	0.16
P24-3n	6.8	36.7	32.4	22.3	98.2	0.36
P24-3ml	10.3	31.9	25.9	28.3	97.5	0.07
p24-3mc	7	27.5	29.5	37	101	0.18
P17-1n	11.6	26.5	26.9	35.4	100.4	0.15
P17-1ml	12.3	28.9	28.2	30.0	99.4	0.53
p17-1mc	10.0	32.0	27	30.2	99.2	0.26

Were: n: native peptides; ml: lineal modified peptides; mc: cyclic modified peptides.
TFE: trifluoroethanol

residues 12-22). The presence of an a-helical region, from residues 11 to 19 has been reported (8). ELISA results obtained with p17-1ml suggest that an increased sensitivity may be reached with this modified sequence, which also showed the highest affinity for antibodies in solution, as was determined by competitive immunoassays.

The CD analyses for p17-1 peptides suggest an stabilization of the secondary structure in the modified sequences, in comparison with the native.

The presence of an extended helical conformation in p24 protein, region 197-213 (AMQMLKETINEEAAEWD) was previously reported (2, 4). Peptide p24-1n (196-224) completely overlaps this helical region, and additionally is elongated towards the N- and C- terminus of p24 protein.

According to the immunochemical results peptide p24-1n was the most immunoreactive and specific of all the studied p24 and p17 peptides, suggesting that this sequence actually contain an immunodominant epitope.

The CD analysis of p24-1n in the presence of an a-helix inductor as trifluoroethanol (TFE/H_2O (80:20)) allowed the detection of a conformational change towards a predominant a-helix (42%). These observations support that p24-1n secondary structure corresponds to a nascent helix structure that is stabilized in the presence of fluorinated solvents.

Peptide p24-1ml contains a less extended helical region because some amino acid residues close to the N-terminal end of p24-1n, with high propensity to adopt a helical structure (Glu, Met, Ala, Leu, Lys) are absent in the modified form. The ability for antibody recognition of p24-1ml was lower than that determined for the native sequence.

Peptide p24-2n (287-307) contains an a-helical sequence between residues 294-304 (RDYVDRFYKTL) (2) and the minimal size epitope (22) is localized in this region. Peptide p24-3n (303-336) contains a short helical region between residues 179-186 (QEVKNWMT) (2). The ability for antibodies recognition of the modified linear peptides p24-2ml and p24-3ml in solid and solution phase, was greater than that established for the corresponding native sequences. A similar situation was also observed for peptide p17-1ml.

The low reactivity observed for p24-2n and p24-3n may be explained considering: a- the two distant regions could possibly be a part of a conformational epitope, in agreement with a previous report (2); b- there is a negative influence of the solid support on which the peptides were immobilized (23).

As shown in Table 3, p24-2ml and p24-3ml have an increased α-helix content in comparison with the native sequence. The addition of TFE to p24-2ml showed the conformational change from a predominant b-sheet to an α-helix (40%).

For the cyclic versions of the modified p24-1, p24-2, p24-3 and p17-1 peptides, higher dissociation constants and lower reactiviy in indirect ELISA were observed, suggesting that the cyclization disturbes significantly the native α-helical conformation.

CONCLUSIONS

The results of studying the ability for antibodies recognition of synthetic epitope peptides corresponding to a-helical regions of p24 and p17 have shown that a sequence overlapping residues 196-224 of p24 HIV-1core protein may be a useful antigen for diagnostic assays. Additional studies are necessary to do in order to determine the relevancy of this p24 antigenic sequence for monitoring the evolution of antibody responses during the course of HIV-1 infection. Its secondary structure corresponds to a nascent helix and contains an immunodominant epitope, giving optimal results in solid and solution phase immunoassays.

The reactivity of peptides mimicking short a-helical secondary structures of p24 core protein (287-307, 303-326) and p17 matrix protein (17, 27) was improved by adding short non immunogenic chains on both N- and C-terminus. These modifications enhance the immobilization onto the solid support, and allow more accesibility to the minimal epitopes by specific antibodies, in solution.

This work emphasizes the relevancy of combining structural and immunological studies for the development of synthetic antigens.

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