An Alpha Helix Conformationally Restricted Peptide Is Recognized by Cervical Carcinoma Patients' Sera

Julio C. Calvo,*,† Katia C. Choconta,† Diana Diaz,† Oscar Orozco,‡ Maria M. Bravo,‡ Fabiola Espejo,† Luz M. Salazar,† Fanny Guzman,† and Manuel E. Patarroyo†,§

Fundación Instituto de Inmunología de Colombia (FIDIC), Carrera 50 No. 26-00, Bogotá, Colombia, Instituto Nacional de Cancerología, Calle 1 No. 9-85, Bogotá, Colombia, and Universidad Nacional de Colombia, Bogotá, Colombia

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Human papillomavirus type 16 (HPV-16) represents the major cervical carcinoma associated virus among women, especially in Colombia. It has thus become important to develop reliable inexpensive tests for detecting the presence of this virus. It has been shown that HPV16-E7 oncoprotein structural features have three α-helical structures and a loop-like structure. The hydrazone link approach was used to mimic helix secondary substructures. Sera from women with invasive cervical carcinoma were tested against conformationally restricted peptides and their respective linear peptides to identify conformational epitopes. One peptide that was conformationally restricted to an α-helix showed very strong positive reaction with sera from women having invasive cervical carcinoma; there was no reaction with sera from patients with other carcinomas, children, or healthy women. NMR studies confirmed this peptide's α-helical structure. The observation that constrained protein substructure peptidomimetics can identify new conformationally sensitive antibodies in cervical carcinoma patients' sera is very important, since these antibodies are almost all generated by native proteins, providing a new selection of antibodies for diagnostic and vaccine studies.

1. Introduction

The human papillomaviruses (HPVs) represent a family of viruses causing various proliferative diseases in the infected epithelium. More than 20 HPVs are associated with oral and anogenital squamous mucosal epithelium infections. These mucosal-associated viruses are subdivided into "low-risk" (e.g. HPV-6), associated with benign genital warts, and "high-risk" (e.g. HPV-16), associated with lesions such as intraepithelial neoplasia, which can progress to anogenital cancer.[1] HPV-16 early gene regions 6 and 7 (E6 and E7) remain intact after integration into host DNA. They are actively expressed in carcinoma cells, leading to E6 and E7 HPV-transforming protein production which, together, can immortalize human fibroblasts.[2] HPV-16 E7 is a 98-residue nuclear phosphoprotein[3] having an amino acid sequence and functional similarity to the adenovirus E1A protein and the large SV40 tumor antigen.

Cervical cancer is strongly associated with high-risk HPVs and is the second leading cause of death from cancer in women worldwide. This occurs despite the wide availability of cytologic screening (e.g. Papanicolaou smear). The probable causes for this problem have been recently summarized, including the high Papanicolaou smear false-negative rate.[4] HPV types 16, 18, 31, and 45 are most commonly associated with invasive cervical cancer (INCA); they are present in about 80% of women with this type of neoplasia, HPV-16 being the most common. The prevalence of a strain can vary with each location.[5] Low- and high-risk HPV infections are common and most of them are transient, regressing spontaneously over a period of several months, whereas a small number persist and may progress to cervical intraepithelial neoplasia (CIN) and INCA after a considerable period of time.[6,7]

The strong association between the presence of mucosal HPV types and the development of cervical cancer has boosted interest in HPV serology for use in epidemiology and as a diagnostic or prognostic marker.[8] Several groups have reported significantly higher HPV-16 E7 antibody prevalence in cervical carcinoma patients than in controls[9] and have described postoperative follow-up of antibody response to HPV-16 peptides in cervical cancer.[10] Neither synthetic peptides, fusion proteins, nor native proteins represent ideal antigens for serological assays, as only nonconformational epitopes become presented.[11−14] Although proteins display more epitopes, they also create more opportunities for cross-reactivity, thus complicating analysis.[15] Mapping E7 reactivity to specific E7 linear peptides was unsuccessful, suggesting that natural or induced E7 reactivity in human serum is commonly directed at E7 conformational epitopes.[16]

Recent approaches to serology have been focused on new strategies that could improve this situation by evaluating possibilities for using conformationally restrained peptides instead of recombinant proteins in vaccine synthesis. Satterthwait et al.[17,18] have developed a novel strategy for constraining peptides to mimic native protein substructures that, when compared with linear peptides, show enormous affinity enhancements for antibodies generated by native proteins in ELISA test.[19−21]

On the basis of Satterthwait's novel strategy for constraining peptides to mimic native protein substruc-

* Corresponding author. FAX: 57-1-4815269. E-mail: jc_calvo@fidic.org.co or jccalvo@eudoramail.com.
† FIDIC.
‡ Instituto Nacional de Cancerología.
§ Universidad Nacional de Colombia.

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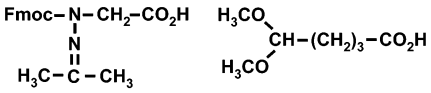
(1a)

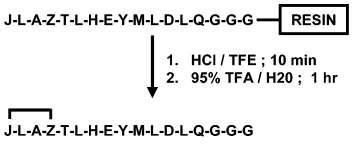
Peptide	Sequence	Residues
Linear-1	MHGDTPTLHEYMLDLQPETT	1-20
Helix-1	JLAZ-TLHEYMLDLQ-GGG	7-16
Linear-2	QLNDSSEEEDEI	27-38
Helix-2	JLAZ-QLNDSSEEEDEI-GGG	27-38
Linear-3	VTFCCKCDSTLRLCVQ	55-70
Loop	VTFCCKCDSTLRLCVQ	55-70

(1b)



Fmoc-Z(Act) linker J linker

(1c)



(1d)

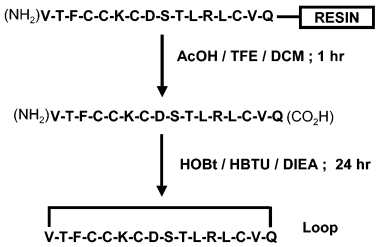


Figure 1. (a) Linear and constrained peptides designed and synthesized for this study. (b) Structure of **J** and **Z** linkers. (c) Insertion of a hydrazone link into a helix-like peptide during solid-phase synthesis. (d) Head-to-tail cycling of loop-like peptide.

tures, peptides were conformationally restricted to an α-helix. The head-to-tail approach was used to synthesize a loop. These two strategies mimicked HPV-16 E7 protein's structural features, as predicted by the Garnier algorithm.[22] Sera from female patients with invasive cervical carcinoma attending the Instituto Nacional de Cancerología de Colombia were tested against these mimetics, presenting sufficient sensitivity for use in high-capacity assays. However, sera from normal women and children did not show any reaction. NMR was used to determine the three-dimensional structure of one (out of the two) α-helix conformationally restricted peptide that reacted strongly to patients' sera.

2. Experimental Section

Design of Conformationally Restricted Peptides. This study was focused on detecting B-cell epitopes on the human papillomavirus type 16 E7 protein by using human sera reactivity against restrained peptides; these were designed on the structural features of the HPV-16 E7 oncoprotein (predicted by the Garnier algorithm). The prediction suggested the presence of three α-helical structures in segments encompassing residues 11−17 (YMLDLQP), 27−38 (QLNDSSEEEDEI), and 75−83 (DIRTLEDLL) and a loop-like structure between 54 and 70 (IVTFCCKCDSTLRLCVQ). The approach used for helix-like peptide synthesis was based on replacing a structure-defining main-chain hydrogen bond (NH···O=C−CHR−NH) with a hydrazone link (N−N=CH−CH_2−CH_2); this was inserted into the peptide sequence during solid-phase synthesis, using an already reported protocol.[17−18] A list of designed and synthesized linear and conformationally restricted peptides is given in Figure 1a. Cysteines for peptide **3** (Figure 1a) were deprotected. The purification process gave a product having the right molecular weight; however, disulfide bond formation could not be detected with the mass spectrometry method used (MALDI-TOF). The hydrazone methodology makes use of two linkers (Figure 1b**)** identified as **J** (5,5-dimethoxypentanoic acid) and Fmoc-**Z** [(1-methylethyliden-2-Fmoc)hydrazinoacetic acid]. **Z** linker was inserted at the end of the target sequence followed by an alanine (A) and a leucine (L) and capped with the **J** linker for mimicking helix peptides. The complete **J**LA**Z** capping sequence was called the "nucleation site" (Figure 1c**)**. Hydrazone link formation and the subsequent cycling employed to obtain the nucleation site occurred because of the reaction between acetal (**J**) and hydrazine (**Z**) derivatives. The loop-like peptide was synthesized on a chlorotrityl resin following the head-to-tail cycling procedure (Figure 1d) described by Zimmer et al.[23]

Linear Peptide Synthesis. The linear and helix-like peptides were synthesized by solid-phase synthesis in glass vessel reactors on Fmoc-MBHA Rink resin with 0.64 mequiv/g (NovaBiochem) substitution following the Fmoc (fluorenylmethyloxycarbonyl) strategy.[24]

Helix-like Peptide Synthesis. Insertion of the hydrazone link into the linear sequence required the prior preparation of **J** linker, Fmoc-**Z**(Act) linker, and Fmoc-alanine chloride. **J** linker (as a methyl ester) and Fmoc-**Z** linker were prepared as described by Cabezas and Satterthwait.[18] Fmoc-alanine chloride was prepared by refluxing the Fmoc-amino acid with $SOCl_2$ in dichloromethane for 15 min and rotary evaporating solvent and excess $SOCl_2$. Fmoc-**Z**(Act)-OH was coupled to peptide-resin by activating this linker with *N*-hydroxybenzotriazole (HOBt) and PyBOP (NovaBiochem). The following residue, Fmoc-alanine chloride, was dissolved in dry dichloromethane and then added to the **Z**-peptide-resin, mixed for one minute before adding diisopropylethylamine (DIEA), and allowed to proceed for 15 min. Fmoc-Leu-OH and **J** linker were coupled by activating them with HOBt/PyBOP. The assembled peptide was cycled with catalytic quantities of hydrochloric acid in 20% 2,2,2-trifluoroethanol (TFE) in dichloromethane; the reaction was allowed to proceed for 15 min. Helix-like peptides were cleaved from the MBHA Rink resin after cycling; protecting groups were removed by treatment with 95% trifluoroacetic acid in water for 1 h, precipitated with ether, and then washed three times with ice-cold ether.

Loop-like Peptide Synthesis. The loop-like peptide was synthesized by solid-phase synthesis in a glass vessel reactor on a chlorotrityl resin with 1.35 mequiv/g substitution (NovaBiochem), following previously described procedures.[23] The protected peptide, having free N- and C-terminals, was dissolved in acetonitrile and cycled by adding 3 equiv of HOBt, 3 equiv of HBTU, and 1% (v/v) DIEA. The reaction was allowed to proceed for 24 h; the solution was concentrated by rotary evaporation and the crude cyclic peptide was deprotected.

Crude synthesized products were purified by preparative RP-HPLC, using a preparative Vydac C18 210TP1022 column (2.2 × 25 cm) in a 15%−70% B linear gradient for 45 min (A, 0.5% TFA in water; B, 0.5% TFA in acetonitrile). Peptide purity was judged by HPLC (analytical Vydac 218TP54 column; 0%-70% B linear gradient for 30 min) and molecular mass confirmed by MALDI-TOF MS.

Human Sera. Human positive sera were obtained from 28 women having cervical carcinoma attending the Department of Gynecology, of the Instituto Nacional de Cancerologia, in Bogotá, Colombia. HPV-positive sera (HPV+) consisted of sera taken from 22 women with cervical cancer (HPV16 E7 positive by immunoblot) and six women having cervical cancer who were negative to the protein. Negative controls consisted of HPV-negative control sera (HPV−) obtained from 10 women who were reported as having normal cytology by the Liga de Lucha Contra el Cancer, Bogotá, Colombia, plus sera from five randomly selected girls also attending the clinic.

ELISA. For the assay, 10 μg of the corresponding peptide per milliliter diluted in 0.1 M carbonate−bicarbonate buffer, pH 9.6, was placed in microtiter-plate wells and incubated overnight at 4 °C. The ELISA plate's antigen-coated wells were then washed with PBS containing 0.5% Tween-20 (PBS-T). The wells were blocked with 100 μL 1% BSA in PBS at 37 °C for 30 min and then washed with PBS-T. Human sera samples

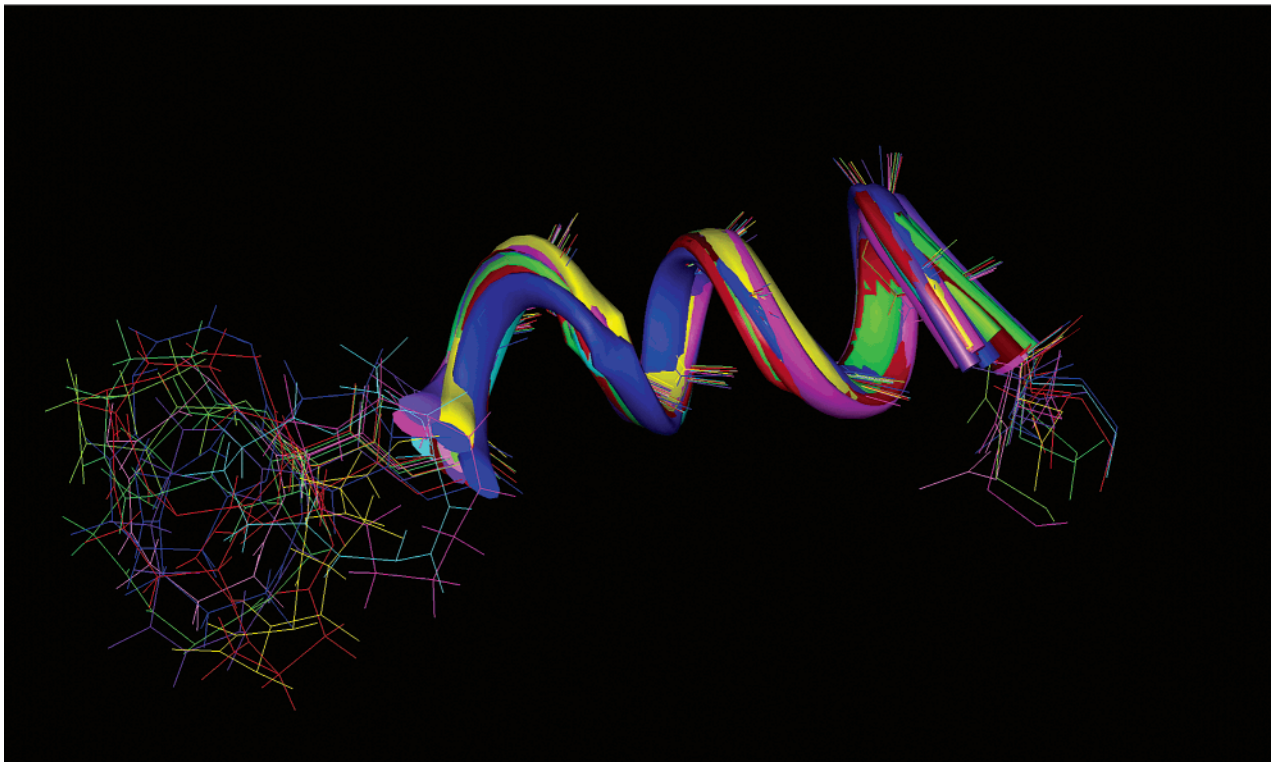


Figure 2. Ribbon representation of the helix-1 peptide structure.

(diluted sequentially from 1:400 to 1:25 600) were then placed in microtiter-plate wells, incubated at 37 °C for 60 min, and then washed with PBS-T. The microtiter-plate wells were then incubated with 100 μL goat anti-human IgG peroxidase conjugate (Sigma), diluted 1:5000 in PBS-T, incubated at 37 °C for 60 min, washed with PBS-T, and finally incubated with the substrate (solution A, TMB; solution B, H_2O_2, 1:1 v/v, from Kirkegaard & Perry) for 15 min. The reaction was stopped with 100 μL of 1 N H_3PO_4, and optical densities were determined at 450 nm wavelength, using an ELISA plate reader.

NMR Spectroscopy. A 8.0 mg portion of helix-1 peptide (showing higher reaction with human sera) was dissolved in 600 μL of a 70:30 H_2O/TFE solution for NMR spectroscopy analysis. All NMR spectra were recorded at 25 °C on a Bruker DRX 600 spectrometer. Spectra were processed using xwin-NMR and Felix software using a Silicon Graphics workstation. ^{1}H assignments and peptide secondary structure determination were achieved from standard procedures described by Wüthrich.[25] DQF−COSY double-quantum-filtered correlation spectroscopy[26] and homonuclear Hartman−Hahn transfer experiment, using Mlev17 TOCSY[27] (total correlation spectroscopy) pulse sequences, were used to assign spin systems. Nuclear Overhauser effect spectroscopy NOESY[28] was used for sequence assignment. The water signal (4.75 ppm) was used as a proton chemical displacement reference. The $^3J_{HN-H\alpha}$ coupling constants were measured by using multiplet-line separation in DQF−COSY spectra cross-peaks or in 1D spectra.

Structure Calculations. An Indigo workstation (Silicon Graphics) running Accelrys software was used to analyze the obtained data. Cross-peak intensity was classified as being strong, medium, and weak, corresponding to 1.8−2.8, 2.8−3.5, and 3.5−5.0 Å ranges, respectively. The ϕ dihedral angle constraints were taken into account only when the value of derived $^3J_{HN-H\alpha}$ coupling constants was less than 6 Hz, to which a $-70° \pm 30°$ ϕ angle range was assigned. Discover software was used to generate a family of 50 structures. Calculations were repeated several times until one having the minimum of distance and angle restraint violations and the least rmsd (root-mean-square deviation), respecting consensus lesser energy structures, was obtained. PROCHECK[29] software was used to check refined structure geometry, especially the ω dihedral angle and the most probable zone within the Ramachandran plot. Structures having reasonable geometry and few violations were then selected.

3. Results and Discussion

Peptide Synthesis. Peptide purity was higher than 95%, as judged by RP-HPLC analysis. Peptides showed the correct molecular weight as measured by MALDI-TOF mass spectrometry with a Bruker Protein TOF instrument (Supporting Information). CD spectra in water and 30% TFE/water were collected on linear-1 peptide, revealing a random structure trend (Supporting Information).

NMR Assignments. Complete $d_{\alpha N}$ sequential NOE's were found for helix-1 peptide. Intraresidual signal intensity greater than that of $d_{\alpha N}$ sequential signals and the presence of strong d_{NN} cross-peaks indicated that there was a significant population of conformations in the $\phi\psi$ space α region. A set of sequential and medium range NOE connectivities and $^3J_{HN-H\alpha}$. NOESY cross-peaks (Supporting Information), correlating sequentially adjacent d_{NN}, $d_{\alpha N}$, and $d_{\alpha\beta}$ residues, were observed in all spin systems. Low $^3J_{HN-H\alpha}$ (6 Hz), numerous short-range sequential and medium range NOE's between L_2 and Q_{10} residues, strong $d_{\alpha N}$ and medium-range $d_{\alpha N}(i,i+2,i+3,i+4)$ and $d_{\alpha\beta}(i,i+3)$ NOE's indicated the presence of a three-dimensional α-helix structure extending from residue T_1 to Q_{10}.

Structure Calculations. The database used to derive the helix-1 peptide three-dimensional structure contained a total of 109 NOE constraints (including short, medium, and long range), 12 ω dihedral angle constraints, and five ϕ dihedral derived from $^3J_{HN-H\alpha}$. All peptide bonds were forced to trans and Cα quirality to L during calculations. A nonbonded 6 Å cutoff distance was used. The 17 best fitting conformers (Figure 2) were selected from the 50 remaining from the last set of constraints obtained following a series of preliminary

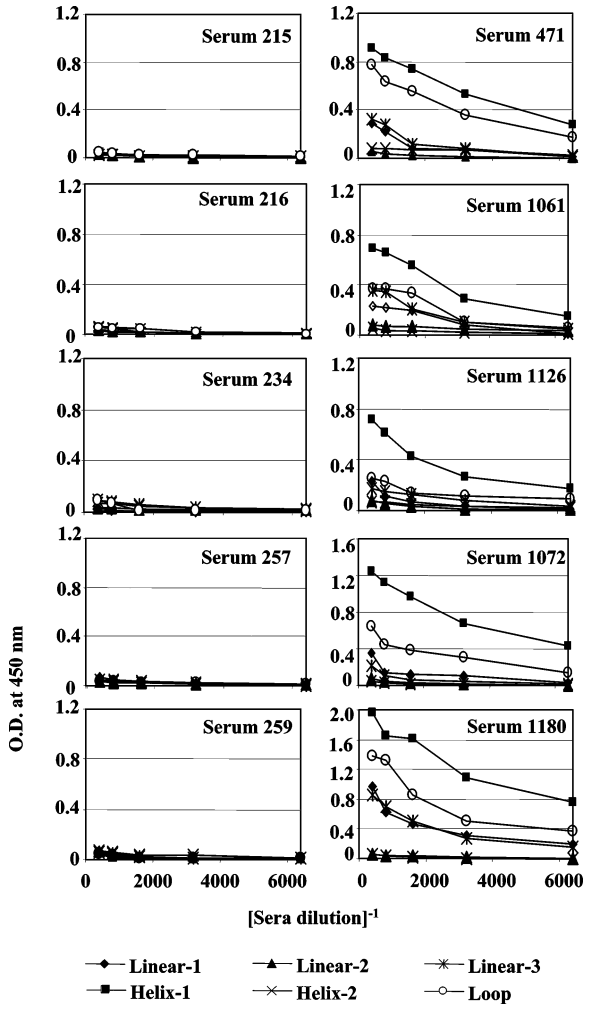


Figure 3. Reaction of human sera against linear and corresponding conformationally restricted peptides; the left column represents sera from healthy women and the right column sera from women having cervical cancer.

runs. These conformers had no distance violation greater than 0.3 Å and no angular constraint violation greater than 2.0°. The 17 structures' backbone atoms' root mean-square-deviation (rmsd) versus consensus structure rmsd was 0.36 Å. The NOE connectivity pattern and coupling constraints smaller than 5 Hz showed an α-helix structure from T_1 to Q_{10}.

Serological Studies. A preliminary ELISA assay was carried out to determine those regions involved in conformational recognition of antibodies directed against the HPV-16 E7 native protein present in the women's sera. Five selected sera (showing reactivity against HPV16 E7 recombinant protein expressed in *Escherichia coli*) from women having cervical cancinoma and five sera from healthy women (reported as having normal cytology) were tested. The level for seropositivity was preassigned as the mean negative control absorbance value plus three times the standard deviation. Human sera were diluted from 1:400 to 1:6,400. Sera numbers 215, 216, 234, 257, and 259 came from healthy women, while sera numbers 471, 1061, 1072, 1126, and 1180 were from women with cervical carcinoma. Normal sera showed no reaction against linear or restricted peptides. On the contrary (Figure 3), sera 471, 1061, 1072, 1126, and 1180, from patients with cervical carcinoma, showed a very strong reaction to helix-1, a

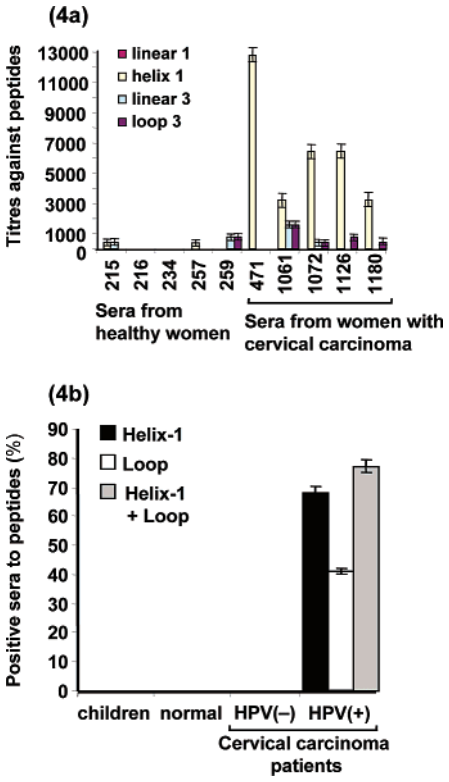


Figure 4. (a) Titers of human sera against linear and restricted peptides. (b) Specific recognition of helix-1 and loop peptides by patients having cervical cancer.

strong reaction with loop-like peptide, a weak reaction to their corresponding linear-1 and linear-3 peptides, and no reaction to helix-2 and its corresponding linear-2 peptide. This general behavior reflects the absence of HPV16 E7 protein antibodies in healthy women, while sera from women with cervical cancer strongly reacted against constrained peptides (Figure 3). All serum samples were tested as before at dilutions ranging from 1:400 to 1:25 600 to identify the cutoff dilution point for designing the scanning test. Second helix-like and corresponding linear peptides were discarded since they did not show any reaction to positive or negative sera samples (Figure 4a). The higher 1:1000 cutoff point in human sera dilution was set because it detected the presence of conformational epitopes better and decreased the probability of false positives. Titration for peptide sensitivity revealed that a 1:1000 dilution cutoff also corresponded to low sensitivity for HPV16 E7 linear peptides. A 1:1000 patient sera dilution and 1:15 000 goat anti-human IgG peroxidase dilution was therefore used for the sera screening assay.

Specific Serological Recognition of Helix-1 Peptide. A total of 68% of patients (15/22) were helix-1 positive at the high dilution sera screening used and 41% of patients (9/22) were loop-like peptide positive. Total screening rose to 77% of patients (17/22), since two helix-1 negative patients were positive to loop-like peptide (Figure 4b). There was high specificity and sensitivity of sera from women with cervical cancer to the small 10-mer helix-1 peptide. These results are very promising in terms of serological cervical cancer diagnosis, since no false positive reactivity was observed.

The observation that constrained peptidomimetics of protein substructures can routinely identify new con-

formationally sensitive antibodies in sera that go undetected by unconstrained peptides is very important. It signals the wide prevalence of antibodies generated by native proteins that have been ignored for want of a detection method. Since these antibodies are almost certainly generated by native proteins, they provide a new and much better selection of antibodies for diagnostic and vaccine studies. The high affinity of helix-1 peptide for sera from patients with cervical cancer suggested that this peptide adopted conformations mimicking the bound conformer to a much better degree than linear peptide.

Restricted Peptide Approach. The hydrazone link approach was used in this study to conformationally restrain those sequences showing helical conformation preference in prediction analysis. It has been shown that this link stabilizes an α-helix conformation in water. This approach did not reveal any false positive reaction with patients' sera having cervical cancer negative to HPV type 16 (six sera samples), healthy women (10 sera samples), or children (five sera samples). ELISA was used in previous serological tests by other groups on sera samples from women with invasive cervical cancer and controls to reveal antibodies to HPV16 virus-like particles. The sera had been previously tested for antibodies titers to HPV16 E6 and E7 oncoproteins. However, there was no correlation between HPV16 E6 or E7 and VLPs sera reactivity. HPV 16 virus-like particles (VLPs) have been used as a screening test for invasive cervical cancer, but this test has been limited by the high prevalence of HPV+ women. Increasing the sensitivity of VLP serology decreased its specificity, resulting in many false-positive results. These results have been justified through patients probably having been previously exposed to HPV 16 but not having had the disease.[15]

The reactivity of patients' sera to HPV16 E7 protein was estimated in recent work by nonradioactive immunoprecipitation assay and by protein- and peptide-based immunoassays. It was observed that no single assay has been particularly sensitive for E7 reactivity or predictive for cervical cancer. They found that mapping E7 reactivity to specific E7 peptides was unsuccessful and suggested that natural or induced E7 reactivity in human serum is commonly directed toward E7 protein-conformational epitopes.[16]

Polymerase chain reaction (PCR) technology has been used to detect HPV DNA in cervical lesions.[30−31] Although PCR analytical sensitivity is higher than ELISA, it varies in terms of sensitivity and specificity in clinical samples and between different laboratories.[32] Besides, its simplicity and the low cost of its serological analysis makes ELISA a useful and cheaper test in diagnosis. It has been reported that antibody level against HPV E6 and E7 increases in the sera of patients having advanced stages of cervical cancer and are correlated with the disease's clinic stage.[33] Our work emphasizes how conformationally restricted peptides can be used to mimic these epitopes with successful results, making it a simple, economical, and reliable test for early cervical carcinoma diagnosis.

Conclusion

The observation that constrained peptidomimetics can routinely identify new conformationally sensitive antibodies in sera that go undetected by unconstrained peptides is very important, since they can be used with some other peptides from the most prevalent "high-risk" HPVs as a rapid, cheap, reliable screening methodology. Previously, just the cumbersome, expensive Papanicolaou smear was available as the method for cervical cancer diagnosis. Overall, this study demonstrates the enormous effect that conformational restriction can have on the antigenicity of a short, synthetic peptide. It also shows hydrazone linker stability and makes clear the role that restricted peptides can play in improving antigenicity and in identifying antibodies that previously went undetected for want of linear peptides allowing the design of specific conformational epitopes for diagnosis or vaccine development.

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Supporting Information Available: HPLC and MS spectra of all peptides, the CD spectra of linear-1 peptide, and the NOESY NMR spectra and NOE assignments of helix-1 peptide. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Gissmann, L.; Boshart, M.; Durst, M.; Ikenberg, H.; Wagner, D.; Zur Hausen, H. Presence of human papillomavirus in genital tumours. *J. Invest. Dermatol.* **1984**, *82*, 26s−28s.
(2) Munger, K.; Phelps, W. C. The human papillomavirus E7 protein as a transforming and transactivating factor. *Biochim. Biophys. Acta* **1993**, *1155*, 111−123.
(3) Smotkin, D.; Wettstein, F. O. The major human papillomavirus protein in cervical cancers is a cytoplasmic phosphoprotein. *J. Virol.* **1987**, *61*, 1686−1689.
(4) Wright, T. C., Jr.; Denny, L.; Kuhn, L.; Pollack, A.; Lorinez, A. HPV DNA testing of self-collected vaginal samples compared with cytologic screening to detect cervical cancer. *JAMA* **2000**, *283*, 81−86.
(5) Combita, A. L.; Touze, A.; Bravo, M. M.; Orozco, O.; Coursaget, P. Antibodies against 7 high risk HPV VLP in invasive cervical cancer patients in Colombia. 18th International Papillomavirus Conference. Barcelona, 2000, Poster 417.
(6) Franco, E. L.; Villa, L. L.; Sobrinho, J. P.; Prado, J. M.; Rousseau, M. C.; Desy, M.; Rohan, T. E. Epidemiology of acquisition and clearance of cervical human papillomavirus infection in women from a high-risk area for cervical cancer. *J. Infect. Dis.* **1999**, *180*, 1415−1423.
(7) Kadish, A. S.; Timmins, P.; Wang, Y.; Ho, G. Y. F.; Burk, R. D.; Ketz, J.; He, W.; Romney, S. L.; Johnson, A.; Angeletti, R.; Abadi, M.; T. A. E. C. D. C. Consortium. Regression of cervical intraepithelial Neoplasia and loss of human papillomavirus (HPV) infection is associated with cell-mediated immune responses to an HPV type 16 E7 peptide. *Cancer Epidemiol. Biomarkers Prev.* **2002**, *11*, 483−488.
(8) Silins, I.; Avall-Lundqvist, E.; Tadesse, A.; Jansen, K. U.; Stendahl, U.; Lenner, P.; Zumbach, K.; Pawlita, M.; Dillner, J.; Frankendal, B. Evaluation of antibodies to human papillomavirus as prognostic markers in cervical cancer patients. *Gynecol. Oncol.* **2002**, *85*, 333−338.
(9) Müller, M.; Viscidi, R. P.; Sun, Y.; Guerrero, E.; Hill, P. M.; Shah, F.; Bosch, X.; Muñoz, N.; Gissmann, L.; Shah, K. V. Antibodies to HPC-16 E6 and E7 proteins as markers for HPV-16-associated invasive cervical cancer. *Virology* **1992**, *187*, 508−514.
(10) Lehtinen, M.; Leminen, A.; Kuoppala, T.; Tiikainen, M.; Lehtinen, T.; Lehtovirta, P.; Punnonen, R.; Vesterinnen, E.; Paavonen, J. Pre- and posttreatment serum antibody responses to HPV 16 E2 and HSV2 ICP8 proteins in women with cervical carcinoma. *J. Med. Virol.* **1992**, *37*, 180−186.
(11) Kanduc, D.; Lucchese, A.; Mittelman, A. Individuation of monoclonal anti-HPV16 E7 antibody linear peptide epitope by computational biology. *Peptides* **2001**, *22*, 1981−1985.
(12) Baay, M. F. D.; Duk, J. M.; Burger, M. P. M.; de Bruijn, H. W. A.; Stolz, E.; Herbrink, P. Follow-up of antibody responses to human papillomavirus type 16 E7 in patients treated for cervical cancer. *J. Med. Virol.* **1995**, *45*, 342−347.

(13) Rocha-Zabaleta, L.; Jordan, D.; Pepper, S.; Corbitt, G.; Clarke, F.; Maitland, N. J.; Sanders, C. M.; Arrand, J. R.; Stern, P. L.; Stacey, S. N. Differences in serological IgA responses to recombinant baculovirus-derived human papillomavirus E2 protein in the natural history of cervical neoplasia. *Br. J. Cancer* **1997**, *75*, 1144−1150.
(14) Dillner, J. The serological response to papillomaviruses. *Cancer Biol.* **1999**, *9*, 423−430.
(15) Sun, Y.; Eluf-Neto, J.; Bosch, F. X.; Munoz, N.; Walboomers, J. M.; Meijer, C. J.; Shah, K. V.; Clayman, B.; Viscidi, R. P. Serum antibodies to human papillomavirus 16 proteins in women from Brazil with invasive cervical carcinoma. *Cancer Epidemiol. Biomarkers Prev.* **1999**, *8*(10), 935−940.
(16) Malcolm, K.; Meschede, W.; Pawlita, M.; Koutsky, L. A.; Frazer, I. H. Multiple conformational epitopes are recognized by natural and induced immunity to the E7 protein of human papillomavirus type 16 in man. *Intervirology* **2000**, *43*(3), 165−173.
(17) Chiang, L. C.; Cabezas, E.; Calvo, J. C.; Satterthwait, A. C. The conformational restriction of synthetic peptides to the alpha helix, reverse turn and loops with covalent hydrogen bond mimics. In *Peptides: Biology and Chemistry (Proceeding of the Chinese Peptide Symposium 1992)*; Du, Y. C., Ed.; ESCOM: Leiden, 1993; pp 204−206.
(18) Cabezas, E.; Satterthwait, A. C. The hydrogen bond mimic approach: Solid-phase synthesis of a peptide stabilized as an α-helix with a hydrazone link. *J. Am. Chem. Soc.* **1999**, *121*, 3862−3875.
(19) Calvo, J. C.; Perkins, M.; Satterthwait, A. C. The identification by chemical synthesis of a conformational epitope on merozoite surface protein 1 of P. falciparum malaria. In *Peptides: Chemistry, Structure and Biology (Proceedings of the 13th American Peptide Symposium)*; Hodges, R. S., Smith, J. A, Eds.; ESCOM: Leiden, 1994; pp 725−726.
(20) Calvo, J. C.; Chocontá, K. C.; Diaz, D.; Orozco, O.; Espejo, F.; Guzmán, F.; Patarroyo, M. E. Preliminar mapping of conformational epitopes on the HPV-16 E−7 oncoprotein. In *Peptides: Frontiers of peptide science (Proceedings of the 15th American Peptide Symposium)*. Tam, J. P., Kaumaya, P. T. P., Eds.; Kluwer Academic Publ.: Dordrecht, 1999; pp 819−820.
(21) Cabezas, E.; Wang, M.; Parren, P. W. H. I.; Stanfield, R.; Satterthwait, A. C. A structure-based approach to a synthetic vaccine for HIV-1. *Biochemistry* **2000**, *39*, 14377−14391.
(22) Garnier, J.; Osguthorpe, D. J.; Robson, B. Analysis and implication of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* **1978**, *120*, 97−120.
(23) Zimmer, S.; Hoffmann, E.; Jung, G.; Kessler, H. Head-to-tail cyclization of hexapeptides using different coupling reagents. *Liebigs Ann. Chem.* **1993**, 497−501.
(24) Carpino, L. A.; Han, G. Y. The 9-fluorenylmethoxycarbonyl function, a new base-sensitive amino-protecting group. *J. Am. Chem. Soc.* **1970**, *92*, 5748−5749.
(25) Wüthrich, K. *NMR of proteins and nucleic acids*; Willey & Sons: New York, 1986; 292 pp.
(26) Rance, M.; Sorensen, O. W.; Bodenhausen, G.; Wagner, G.; Ernst, R. R.; Wüthrich, K. Improved spectral resolution in COSY ^{1H}-NMR spectra of proteins via double quantum filtering. *Biochem. Biophys. Res. Commun.* **1983**, *117*, 419−485.
(27) Bax, A.; Davis, D. G. MLEV-17 based two-dimensional homonuclear magnetization transfer spectroscopy. *J. Magn. Reson.* **1985**, *65*, 355−360.
(28) Jeener, J.; Meier, B. H.; Bachman, P.; Ernst, R. R. Investigation of exchange process by two-dimensional NMR spectroscopy. *J. Chem. Phys.* **1979**, *71*, 4546−4553.
(29) Laskowski, R. A.; MacArthur, M. W.; Moss, D. S.; Thornton, J. M. Procheck a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* **1993**, *26*, 283−291.
(30) Wang-Johanning, F.; Lu, D. W.; Wang, Y.; Johnson, M. R.; Johanning, G. L. Quantitation of human papillomavirus 16 E6 and E7 DNA and RNA in residual material from thinprep Papanicolaou tests using real-time polymerase chain reaction analysis. *Cancer* **2002**, *94*, 2199−2210.
(31) Venturoli, S-; Cricca, M.; Bonvicini, F.; Giosa, F.; Pulvirenti, F. R.; Galli, C.; Musiani, M.; Zerbini, M. Human papillomavirus DNA testing by PCR-ELISA and hybrid capture II from a single cytological specimen: Concordance and correlation with cytological results. *J. Clin. Virol.* **2002**, 177−185.
(32) Kulasingam, S. L.; Hughes, J. P.; Kiviat, N. B.; Mao, C.; Weiss, N. S.; Kuypers, J. M.; Koutsky, L. A. Evaluation of human papillomavirus testing in primary screening for cervical abnormalities: Comparison of sensitivity, specificity, and frequency of referral. *JAMA* **2002**, *288*, 1749−1757.
(33) Rosales, R.; Lopez-Contreras, M.; Cortes, R. R. Antibodies against human papillomavirus (HPV) type 16 and 18 E2, E6 and E7 proteins in sera: Correlation with presence of papillomavirus DNA. *J. Med. Virol.* **2001** *65*, 736−744.

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