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PRESENCE OF ANTIBODY REACTIVE WITH SYNTHETIC PEPTIDE DERIVED FROM L2 OPEN READING FRAME OF HUMAN PAPILLOMAVIRUS TYPES 6b AND 11 IN HUMAN SERA

A. SUCHÁNKOVÁ[1], O. RITTER[1], I. HIRSCH[1], V. KRCHŇÁK[2], Z. KALOŠ[3], E. HAMŠÍKOVÁ[1], B. BŘICHÁČEK[1], V. VONKA[1]

[1]Department of Experimental Virology, Institute of Sera and Vaccines, Prague, [2]Pharmaceuticals, Prague, and [3]Department of Dermatology and Venerology, Central Military Hospital, Prague, Czechoslovakia

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Summary. — Nine oligopeptides corresponding to segments of different open reading frame (ORF) proteins of human papillomavirus (HPV) 6b and HPV-16 were prepared and tested for reactivity with human sera in enzyme-immunoassay (ELISA). Of these only heptadecapeptide derived from L2 ORF of HPV-6b, and encoded also by L2 ORF of HPV 11, was reactive with some human sera. Over 400 human sera of different origin were tested for the presence of antibody to this antigen. While less than 15% of sera from healthy subjects or cervical carcinoma patients were found antibody positive, sera from the majority of *condylomata accuminata* (CA) patients were reactive. The antibody titres varied from 1 : 10 (initial serum dilution) to 1 : 80; in this respect there was no marked difference between sera from CA patients and the other subjects. The prevalence of antibody was higher among promiscuous than nonpromiscuous women. This is in line with the assuption that sexual intercourse is the most important route of HPV 6 and 11 transmission.

Key words: *Human papillomavirus* (*HPV*); *synthetic peptides*; *condylomata accuminata*(*CA*) *patients*; *enzyme-immunoassay* (*ELISA*) *test*

Introduction

Availability of type-specific serological tests with human papillomavirus (HPV) would provide efficient tools in epidemiological studies on these viruses and in assessing their roles in human disease. Studies of this kind have been rare (Pfister and zur Hausen, 1978; Kienzler *et al.*, 1983). In general, they have been hampered by lack of type-specifically reactive antigens. There are two major difficulties involved. First, HPV cannot be propagated *in vitro*. Under these conditions virus induced lesions have served as

the only source of viral antigens for serological studies. Unfortunately — and this is the second limitation — sufficient working amounts of viral materials can only be obtained from some sorts of HPV lesions, especially from plantar and common warts. This has restricted efficient serological work to a few HPV types excluding those most significantly involved in human cancer (Gissmann, 1984). Also the recently developed system utilizing *in vivo* replication of HPV in nude mice xenografted with infected human tissues (Kreider *et al.*, 1987) seems to be confined to certain HPV types and strains. Thus, efforts to categorize HPV serotypes have been quite limited so far and it is not yet clear whether and to what extent the HPV typing based on DNA homology (Coggins and zur Hausen, 1979), corresponds with actual antigenic distinctness. However, the advent of recombinant DNA technology and the ensuing knowledge and skills have provided a means of solving the problem. HPV antigens have been successfully expressed in bacteria (Banks *et al.*, 1987) or eukaryotic cells (Bernard *et al.*, 1987) and, based on the recognition of HPV DNA sequences, virus specific oligopeptides have been synthesized (Li *et al.*, 1987). Both genetically engineered and synthetic peptides have been used for generating antibodies specifically reactive with authentic viral proteins present in HPV lesions and HPV-containing cell-lines (Androphy *et al.*, 1987; Doorbar and Gallimore, 1987; Fitzlaff *et al.*, 1987; Komly *et al.*, 1986; Li *et al.*, 1987; Palefsky *et al.*, 1987; Seedorf *et al.*, 1987; Smotkin and Wettstein, 1986; Tomita *et al.*, 1987).

Fusion proteins synthetized in *E. coli* have also been employed for the detection and characterization of antibodies directed against HPV-6b encoded proteins (Jenison *et al.*, 1988). In this study, most of the sera of *condylomata accuminata* (CA) patients have reacted with a HPV-6b L1 fusion protein but not with the homologous region of HPV 16 capsid antigen. Since the complex nature of the proteins used as antigen has led to frequent non-specific reactions and cross-reactivity, their employment in serology has been limited to Western blot analysis demanding preabsorbtion of sera tested.

In our approach, we preferred to use short synthetic peptides instead of of large recombinant proteins as target molecules, with the hope that we would be able to monitor the antigen-antibody reactions directly and to design a usable antibody assay. Having in mind that the most important aspect of HPV serology in aetiological studies on genital cancer is differentiating between "benign", namely HPV 6 and 11, and "malignant" virus types, namely HPV 16 and 18, we selected for the present study some potential antigenic determinants exhibiting a high degree of type specificity.

Materials and Methods

Sera. The first group consisted of sera taken from 10 healthy children aged less than 3 years. A second group were sera from healthy women aged 25—45 years enrolled into the recent prospective study on cervical neoplasia (Vonka *et al.*, 1984*a*; 1984*b*; 1987). For the present investigation the sera were selected according to (i) donor age, and (ii) number of sexual partners, as reported by the women at the enrollment. As described elsewhere (Vonka *et al.*, 1984*b*; 1987) the reliability of answers concerning sexual behaviour of these subjects was validated by demonstrating internal consistency of the information gathered. It should also be recalled that,

when investigating the same collection of sera previously, we found a relation between sexual promiscuity and prevalence of antibody to herpes simplex type 2, another sexually transmitted agent (Suchánková *et al.*, 1985). All of these women were free of any pathological cervical changes at the time of bleeding. Another group of sera were obtained from 21 women with invasive cervical carcinoma (CC), in the most of whom the previous hybridization tests with biopsy specimens had revealed the presence of either HPV 16 or HPV 18 DNA (Krčmář *et al.*, 1988 and unpublished data). A fourth group of sera originated in laboratory personnel (aged 25 to 63 years). Additional serum samples were collected from some donors' regular sexual partners. This selection was based on data obtained in the first series of experiments and on willingness of their partners to cooperate; a total of 26 sera from 13 married couples were thus collected. Finally, sera were obtained in 21 subjects aged 20 to 25 years suffering from CA for prolonged periods of time; they had experienced up to 10 relapses of the disease. All sera were stored at −20°C until investigation.

Synthetic peptides. Utilizing the knowledge of complete DNA sequence of HPV types 1a, 6b, 8, 11, 16, 18 and 33 (Schwarz *et al.*, 1983; Danos *et al.*, 1982; Seedorf *et al.*, 1985; Dartmann *et al.*, 1986; Cole and Streeck, 1986; Fuchs *et al.*, 1986; Cole and Danos, 1987) we searched for potential type-specific antigenic determinants on the basis of hydrophilicity and secondary structure (presence of β-turn) utilizing Chow and Fasman's computer programme (Chow and Fasman, 1978) as modified by Krchňák *et al.*, (1987). A total of nine peptides ranging from 9 to 17 amino acids in length, derived from E6, E7 and L2 open reading frames (ORF) of HPV types 6 and 16 were selected. The amino-acid sequences of the individual probes are given in Table 1. All the sequences are specific relative to the other sequenced HPV types (see above), except peptide No. 8, derived from the carboxyl-terminal part of HPV 6 L2 ORF; this sequence is shared by the closely related HPV 11.

Merrifield solid phase method (Barany and Merrifield, 1980) as modified by Krchňák *et al.* (1988) for multiple flow solid phase peptide synthesis was used for the peptide preparation. The peptides were purified by reverse phase liquid chromatography and prior to use in serological tests were conjugated to bovine serum albumine (BSA) by glutaraldehyde.

Enzyme-immunoassay (ELISA). All assays were performed in 96-well polystyrene microtitre plates (Kohinoor, Gamma, Dalečín, Czechoslovakia). Wells were coated with 100 μl of the peptide conjugate solution in 50 mmol/l carbonate-bicarbonate buffer (pH 9.6) and dried overnight at 37°C. Unoccupied sites were blocked with bovine serum albumine (BSA) used at a concentration of 10 mg/ml in carbonate-bicarbonate buffer. After 1-hr incubation the plates were rinsed five times with washing buffer (0.35 mol/l NaCl, 2,7 mmol/l KCl, 1.5 mmol/l KH_2PO_4, 6.4 mmol/l $Na\ HPO_4$, 0.1% Triton X-100, pH 7.2) and 0.1 ml of serum diluted 1 : 10 (unless stated otherwise) in washing buffer supplemented with BSA as added per well. Each serum dilution was tested in two parallels. After 1-hr incubation at room temperature the plates were repeatedly rinsed with washing buffer and 100 μl of 1 : 2,500 dilution of peroxidase-conjugated Swine anti-human IgG (SwaHu, Sevac, Prague) was added. The reaction was revealed by adding 100 μl of substrate (O-phenylenediamine) at a concentration of 3.7 mmol/l in 35 m mol/l citrate buffer (pH 5.0) containing 0.006% H_2O_2. It was stopped with 2 mol/l sulphuric acid and measured photometrically at 492 nm in Titertec Multiscan MCC 340 (Flow). Absorbance values determined in two parallels were averaged. A negative and a positive control serum, as determined in the first series of experiments (see Results), were included in each test. Glutaraldehyde-treated BSA served as control antigen.

Competition assay. Serum samples diluted 1 : 5 were mixed with an equal volume (150 μl) of either washing buffer or peptide solution containing 480 μg of the peptide per ml. The mixtures were incubated for 1 hr at 37°C and overnight at 4°C, then they were centrifuged at 4000 rev/min for 1 hr, and the supernatants were tested for antibody content. Control and peptide absorbed samples were run in parallel.

Results

Set-up of test

In the first series of experiments different concentrations of all the peptide conjugates used were tested in ELISA against a panel comprising children's sera and sera from laboratory personnel. While tests with E6 and E7 peptides

Table 1. Amino-acid composition of synthetic peptides

No.	HPV type	ORF	Amino acid sequence	Location[1]
1	16	E6	Asp Pro Gln Glu Arg Pro Arg Lys Leu Pro Gln Leu	11 – 22
2	16	E6	Ile Val Tyr Arg Asp Gly Asn Pro Tyr Ala Val	59 – 69
3	16	E7	Ile Asp Gly Pro Ala Gly Gln Ala Glu Pro Asp Arg Ala	38 – 50
4	6	E6	Gly Lys Ile Asn Gln Tyr Arg His Phe	72 – 80
5	6	E7	Leu Gln Pro Pro Asp Pro Val Gly Leu His	15 – 24
6	16	L2	Pro Val Gly Pro Ser Asp Pro Ser Ile Val Ser Leu Val Glu Glu	98 – 111
7	6	L2	Pro Val Ala Pro Ser Asp Pro Ser Ile Val Ser Leu Ile Glu Glu	96 – 110
8	6+11	L2	Met Gly Thr Pro Phe Ser Pro Val Thr Pro Ala Leu Pro Thr Gly Pro Val	411 – 427
9	16	L2	Ser Ile Thr Thr Ser Thr Asp Thr Thr Pro Ala Ile	134 – 135

[1]) Denotes the position of amino-acids in the respective ORF

Table 2. Presence of 6/11-L2 antibody in healthy women aged 25 to 35 and 36 to 45 years

Age group	No. tested	No. reactive with 6/11-L2 antigen
26–35	77	11 (14.2%)
36–45	86	12 (13.9%)
Total	163	23 (14.1%)

derived from either HPV 6b or HPV 16 and L2 peptides from HPV 16 were invariably negative, some of the adult sera gave a positive reaction with HPV 6 L2 heptadekapeptide, derived from the C-half of L2 and shared by HPV 11 L2 (Table 1). The reaction with this peptide, denoted 6/11-L2, was clearly dependent on the serum and antigen dilution. Box titrations of some of the reactive sera, tentatively considered antibody-positive, showed that the highest difference in reactivity to the test and control antigens was achieved at a concentration of 625 ng of the antigen/ml. This coating amount (i.e. 62.5 ng/well) was thereafter used in all experiments. On the basis of the results obtained, a serum was considered positive if the absorbancy detected with test antigen minus absorbancy with control antigen (i.e. glutaraldehyde-treated BSA) exceeded at least 2.0 times the absorbancy value of negative serum with test antigen. This reaction index (RI) of 2.0 was assigned as a cut-off point after testing ten sera from healthy children considered antibody negative. The mean value of RI of HPV6/11-L2 with these sera was 1.046—0.473; the cut-off point chosen just exceeded the 1.99 value of the mean plus 2 SDs.

To demonstrate that anti-6/11-L2 reactivity was directed against the peptide and not the carrier protein, a competition assay was run with 2 sera displaying titres of 1 : 40 and 1 : 80. The antibody titres decreased by a factor of more than 4 and more than 8, respectively, indicating that the single cycle of preabsorption with the peptide resulted in complete block of the reaction.

Table 3. Presence of 6/11-L2 antibody in healthy women reporting various numbers of sexual partners

No. of partners	No. tested	No. reactive with 6/11-L2 antigen
0	4	0
1	89	9 (10.1%)
2–10	54	8 (15.3%)
11–50	37	9 (23.6%)
Total	184	26 (14.1%)

Table 4. Presence of 6/11-L2 antibody in paired sera obtained in 13 married couples

No. of serum pairs tested	No. reactive with 6/11-L2 antigen		
	Both negative	One positive	Both positive
13	7	6	0

Testing sera from healthy individuals

To get some basic information on the distribution of anti-6/11-L2 antibodies, we tested 159 sera selected at random from a collection assembled at the beginning of our prospective study on cervical cancer (Vonka *et al.*, 1984*a*, 1984*b*). The results are shown in Table 2. It can be seen, that irrespective of age, approximately 14% of sera were reactive at the 1 : 10 dilution.

Assuming that sexual intercourse was the main way of HPV 6 and 11 spreading we tried to ascertain the capacity of the test for monitoring the transmission of these viruses. For this purpose two groups of sera were selected. One again consisted of samples collected at the beginning of the prospective study in women reporting various numbers of sexual partners. Data shown in Table 3 indicate that the presence of 6/11-L2 antibody was related to the number of sexual partners. The percentage of promiscuous women (more than 10 partners) possessing 6/11-L2 antibodies was more than twice as high as that of women with only one partner. The difference between women with one partner and women with more than 10 partners is statistically significant ($p < 0.05$).

Another group of sera originated from 13 married couples. The results of the tests are shown in Table 4. Six subjects were antibody positive. Surprisingly enough, in no instance were both sera of any couple found positive.

Testing sera from patients with condylomata accuminata (CA) and cervical cancer (CC)

Finally, sera obtained in 21 CA patients and 21 CC patients were tested. As is evident from Table 5, the CA patients differed markedly from both the healthy subjects (Tables 2 and 3) and CC patients. Sera from more than half of the CA patients possessed 6/11-L2 antibody while only one of the CC patients was reactive. It was of interest to compare the antibody titres in CA patients and in the antibody positive healthy individuals. The data are presented in Table 6. It can be seen that generally the 6/11-L2 antibody levels were low and that between the groups there was no marked difference, although the highest titre (1 : 80) was detected in one CA patient.

Discussion

In the present series of experiments the sero-reactivity of nine synthetic peptides corresponding to type-specific (relative to HPV types 1a, 6b, 8, 11, 16, 18 and 33) segments of different ORF proteins of HPV 6 and 16 were

Table 5. Presence of 6/11-L2 antibody in patients with condylomata accuminata (CA) and cervical cancer (CC)

Diagnosis	HPV typo detected	No. patients	No. reactive with 6/11-L2 antigen
CA	N.T.[1])	21	11 (52%)
CC	16	10	0
CC	18	5	0
CC	neg.[2])	6	1 (17%)

N.T — not tested
neg. — negative results in dot-blot hybridization test with HPV 16 and 18 DNA probes

tested. Only one of the peptides was found reactive with some human sera. This heptadekapeptide had been derived from the C-terminal half of HPV 6b L2 ORF protein; the particular sequence is shared by the closely related HPV 11. After working criteria for seropositivity had been established, more than 400 human sera of diverse origin were tested for the presence of antibody to this peptide, denoted 6/11-L2. The most significant of the serological findings was that the 6/11-L2 reactive antibody was much more frequent in patients with CA, a disease associated predominantly with HPV types 6 and 11 (Gissmann *et al.*, 1983), than in healthy subjects or in patients with either HPV 16 or HPV 18 assocciated cervical neoplasia. Although CA tissues from the present patients were not examined for HPV DNAs, our previous study on Prague CA patients had revealed DNA of HPV 6 or 11, or both, in nearly all biopsies examined (Krčmář *et al.*, 1988; and unpublished data). The present demonstration of HPV 6 antibody in the majority of CA patients is in agreement with a recent report by Jenison et al., (1988). Utilizing genetically engineered peptides corresponding to several HPV type 6 and type 16 open reading frames, these authors were able to demonstrate antibody reactive with HPV 6 L1 antigen in 9 out of 12 patients; this reaction was type specific relative to HPV 16.

Although the data now obtained in CA strongly suggested that the serological test introduced was really capable of detecting a large proportion of past HPV 6 and HPV 11 infections and was specific relative to HPV types 16

Table 6. 6/11-L2 antibody titres in healthy subjects and in patients with condylomata accuminata (CA)

Group	No.	Antibody titre 1 : 10	1 : 20	1 : 40	1 : 80	GMT
Healthy women	24	15	6	3	0	14.1
CA patients	11	7	1	2	1	16.2

and 18, it is not easy to explain all the present findings in an unequivocal manner. It is likely that the high prevalence of 6/11-L2 antibody in CA patients was a consequence of extensive and long-lasting virus replication i these subjects. The higher prevalence of the antibody in promiscuous than non-promiscuous women was apparently due to a high risk of infection, multiple infections and possibly to infections with both HPV 6 and 11. This finding was in line with the assumption that sexual intercourse is the main route of HPV 6 and 11 transmission. The lack of dependence of antibody presence on age (Table 2) did not militate against this concept, because the younger women tended to start their sexual life earlier and to have more sexual partners than the elder ones (Vonka *et al.*, 1984*b*; 1987). The discordant findings in married couples (Table 4) are the least clear portion of our results. These data are at variance with the previous demonstration that HPV infections when monitored by filter hybridization tests, have been usually shared by both partners (Schneider *et al.*, 1987; Wickenden *et al.*, 1988). Several assumptions can be offered as explanation. First, sexual intercourse is not really the predominant way of HPV 6 and HPV 11 virus transmission, which is unlikely. Second, most of those who have experienced genital infection by HPV 6 or HPV 11 and have developed the anti 6/11-L2 antibody do not shed the virus for prolonged periods of time. Third, most subclinical HPV 6 and HPV 11 infections occurring in sexual contacts of infected subjects are not accompanied by development of antibody reactive with the peptide representing only one of many possible immunoreactive determinants of these two viruses, or result in antibody levels below the threshold of the assay. The low antibody levels in most seropositive subjects (Table 6) point to this possibility. It should also be noted that only IgG antibodies were monitored in the present series of experiments. It had been shown previously that in the case of some HPV infections viral IgM antibody was present for prolonged periods of time in the absence of IgG class antibody (Goffe *et al.*, 1966; Morison, 1975). Such subjects would have been missed in the present study. Should any of the latter possibilities be true — which is likely — the corresponding viruses would be much more widely distributed among the studied population than suggested by the present serological findings.

To decide between the different explanations, more information on both the immunology and epidemiology of HPVs is needed.

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