Synthetic peptides derived from E7 region of human papillomavirus type 16 used as antigens in ELISA

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Nine overlapping peptides (20 amino acid) covering the entire sequence of early antigen E7 of human papillomavirus type 16 (HPV-16) were synthesized and tested as antigens with human sera in ELISA. Five of these peptides (no. 1 to 5 counting from the N terminus of the E7 protein) reacted with a pool of sera from HPV-16-infected individuals (as determined by molecular hybridization with their biopsy specimens); one (no. 5) was also reactive with pools of HPV-18- and HPV-6- or 11-infected individuals. Sera from 24 patients with cervical intraepithelial neoplasia (CIN) and from 29 invasive cervical carcinoma (INCA) patients were tested for the presence of antibodies reactive with peptides, no. 1 to 4 covering amino acids 1 to 50 and with peptide no. 5 covering amino acids 41 to 60. Only one of the sera from CIN patients was reactive with peptides no. 1 (amino acids 1 to 20) and no. 4 (amino acids 31 to 50). However, the majority of these sera reacted with peptide no. 5. The occurrence of this antibody was only slightly less frequent in sera from healthy subjects compared to CIN patients. On the other hand, sera from the INCA patients were reactive with the peptides no. 1 to 3 more frequently than the sera from matched control subjects. Positive reactions of sera from INCA patients were most frequently seen with no. 2; 24% of these sera but only 7% of the controls were reactive with no. 2 peptide. The present data suggest that no. 1 to 3 are HPV-16-specific, whereas no. 5 is broadly cross-reactive.

Introduction

To date, more than 56 distinct human papillomavirus (HPV) types have been identified, 22 of which infect the human genital tract (zur Hausen, 1989). HPV types 6 and 11 are most often found in benign condylomas, whereas HPV types 16, 18, 31, 33 and 35 are detected principally in preneoplastic lesions and invasive carcinomas. Of these, HPV-16 is the most prevalent type as demonstrated by molecular hybridization tests. Owing to the preferential association of different HPV types with benign or malignant conditions, it is of great importance for treatment and prognosis to determine the HPV type involved at the appearance of first symptoms of a pathological process. In addition, detection of an HPV type associated with the malignant disease in a healthy subject may identify women at risk of developing cervical neoplasia. No simple and inexpensive method for HPV detection and typing is as yet available for mass screening programmes. An alternative to the molecular hybridization test would be immunoassays capable of detecting type-specific anti-HPV antibodies in sera of patients and healthy subjects. However, HPVs do not grow in tissue cultures and viral antigens for serological tests cannot be prepared on this basis. Until recently, virus-induced benign lesions served as the only source of HPV antigens for serological tests. However, such materials can only be obtained from some types of benign lesions, e.g. common warts, and contain only certain HPV types, this excluding the HPV types associated with cervical cancer. Therefore, other approaches to obtaining antigens for immunoassay are needed. One way is to utilize information about the structure and sequence of HPV DNA for designing type-specific, serologically non-cross-reacting antigens. Synthetic peptides are easier to prepare than the long amino acid structures obtainable by genetic engineering. The feasibility of this approach has recently been shown by Dillner et al. (1989), who demonstrated antibody reactive with a peptide derived from the E2 open reading frame (ORF) of HPV-16, in a majority of 33 patients with cervical intraepithelial neoplasia (CIN), and by Suchánková et al. (1990) who detected antibodies to a heptadecapeptide derived from the L2 ORF of HPV-6b (and shared also by HPV-11) in some human sera and a high prevalence of this antibody in sera from patients
with condyloma acuminatea, a disease associated with HPV types 6 and 11.

In this communication we report on the use of synthetic peptides derived from the E7 antigen of HPV-16 for the detection of antibodies in human sera.

**Methods**

**Sera.** Four serum samples originated from patients with condyloma acuminatea, 24 were obtained from women at various stages of CIN (CIN 1 to III), and 29 were obtained from patients with invasive cervical carcinoma (INCA). Control sera were obtained from 53 age-matched female patients suffering from various non-malignant otorhinolaryngeal diseases. Another control set comprised 44 specimens from children aged 1 to 3 and 9 to 11 years; these sera had been collected during various vaccination studies. All sera were inactivated by heating at 56 °C for 30 min and kept at −20 °C until used.

**Preparation of synthetic peptides.** Peptides were prepared by continuous-flow solid-phase multiple peptide synthesis (Krčňák et al., 1987, 1989) on p-methyl-benzhydrylamine resin using the Fmoc-t-Bu protection strategy. Fmoc groups were removed by piperidine, condensation was performed by HOBr esters, monitoring of the coupling reaction was by bromophenol blue (Krčňák et al., 1988). Side-chain protecting groups were removed by trifluoroacetic acid and completed peptides were split off the resin in liquid hydrogen fluoride. Crude peptides were purified by gel filtration on Sephadex G15. Purified peptides displayed the correct amino acid composition and showed the expected molecular peak in fast atom bombardment mass spectrometry.

**ELISA.** Wells of plastic plates (Nunc) were coated with peptides (2 µg) dissolved in 10 mm-sodium carbonate–bicarbonate buffer pH 9·5. The plates were incubated for 2 h at 37 °C and overnight at 4 °C. Unoccupied sites were blocked with 1% bovine serum albumin (BSA) in sodium carbonate–bicarbonate buffer pH 9·5, for 1 h at room temperature and the plates were washed five times with phosphate-buffered saline (PBS)-Triton X-100 (0·1%), pH 7·2 (washing solution). Serum samples diluted 10-fold, unless stated otherwise, in PBS–Triton X-100, pH 7·2, with 10% BSA, were added and incubated for 1 h at room temperature. After washing five times with the washing solution, 100 µl of peroxidase-labelled swine anti-human IgG (Sevac, diluted 1 : 2500) was added for 1 h. The plates were then washed five times with washing buffer and the reaction was revealed by adding 100 µl of substrate (0·04% orthophenylenediamine, Sigma) in 50 mm-phosphate–citrate buffer pH 5·0. The reaction was stopped by adding 100 µl 2 mol sulphuric acid and the colour was measured at 490 nm using a Dynatech Microplate Reader.

The following synthetic antigens were used: eight peptides consisting of 20 amino acids and one of 18 jointly covering the entire sequence (98 amino acids) of the protein encoded by the E7 ORF of HPV-16 and consecutively overlapping each other by 10 amino acids. The E7 amino acid sequence and locations of the individual peptides are shown in Fig. 1. In addition, two peptides corresponding to amino acid sequences 11 to 30 and 35 to 54 of the E7 ORF of HPV-1, denoted 1/E7-1 and 1/E7-2, respectively, were used as control antigens. To assign a cut-off point between reactivity and non-reactivity in our peptide-based ELISA, we tested sera of 44 children aged 1 to 3 and 9 to 11 years who should not have experienced infection with HPV-16. The cut-off value selected was the mean absorbance value of these sera plus three standard deviations (S.D.). The mean absorbance plus 3 S.D. of six child sera negative for anti-HPV-1 antibodies (Anisimová et al., 1990) by immunoelectron microscopy (IEM) was defined as the cut-off point between positive and negative values for HPV-1 in ELISA.

**Competition assays.** Adsorption of the reactive antibody was done with the homologous peptides (1/E7-1 and 1/E7-2) and lysates of genetically engineered bacteria expressing HPV-16 E7 and E4 fusion proteins (Escherichia coli C600/537 expressing bacteriophage MS2 polymerase–HPV-16 E7 or E4 fusion protein; Jochmus-Kudielka et al., 1989). Serum that strongly reacted with the 16/E7-2 peptide was diluted 1:5 and mixed with an equal amount of (i) peptide (20 µg/0·1 ml) solution in carbonate–bicarbonate buffer supplemented with 10% bovine serum, (ii) buffer without peptide, (iii) lysate of parental C600/537 MS2 strain of E. coli (MS2), (iv) lysate of induced MS2 E7, i.e. genetically engineered E. coli expressing MS2 polymerase–HPV-16 E7 protein or (v) lysate of induced MS2 E4, i.e. genetically engineered E. coli expressing MS2 polymerase–HPV-16 E4 protein. The lysates of MS2 and MS2 E4 served as control antigens. All mixtures with bacterial lysates contained the same amount (approximately 9 mg) of bacterial proteins. The genetically engineered bacteria were kindly provided by Drs L. Gissmann and I. Jochmus-Kudielka and were cultivated and induced by incubation at 42 °C as recommended by Jochmus-Kudielka et al. (1989). The reaction mixtures were incubated for 1 h at 37 °C and overnight at 4 °C, then centrifuged at 4000 r.p.m. for 10 min, and the supernatants were tested for antibody content. Specific antigen-adsorbed and control samples were tested in parallel.

**DNA hybridization.** The presence of HPV DNA was analysed by dot blot hybridization in materials from cervical smears, by in situ hybridization in biopsies from CIN lesions, and by dot blots and/or Southern blots in biopsies from cervical carcinoma. To obtain dot blots, cellular DNA was isolated from clinical samples and 1 to 30 µg of the DNA was spotted on nitrocellulose (NC) filters and fixed by heating. To prepare Southern blots, DNA was digested with EcoRI or BamHI, the DNA fragments were electrophoretically separated, denatured, neutralized, transferred onto an NC filter, and fixed to the filter by heating. HPV (types 6b, 11, 16, 18, and 30) DNA probes were obtained by nick translation (Rigby et al., 1977) using [32P]dCTP. The original materials had been kindly provided by Dr H. zur Hausen (Deutches Krebsforschungszentrum, Heidelberg). Prehybridization and hybridization were done in 6 x SSC at 68 °C and washing was done at the same temperature, the final wash being with 0·5 x SSC/0·2% SDS (stringent conditions). The filters were then autoradiographed for 7 days. In situ hybridization was performed on tissue sections fixed on glass slides as described in previous experiments (Bířcháček et al., 1983). HPV DNA probes (the same as above) were prepared by nick translation of DNAs isolated from pBR322 using a 3H-labelled
deoxynucleotide mix (70000 c.p.m. per section). Hybridization was in 2.4 × SSC/30% formamide at 43 °C for 40 h. The final wash was in 2 × SSC at 63 °C (stringent conditions). The preparations were dipped in photographic emulsion (ORWO K6), dried, exposed for 4 weeks, developed, and evaluated under a light microscope.

Statistics. A standard chi-square test was used.

Results

The peptides prepared were tested in ELISA for their reactivity with human sera. For screening purposes, four serum pools were prepared: (i) one pool of five sera from INCA patients in whose biopsies HPV-16 DNA had been detected; (ii) one pool of five sera obtained in healthy children aged 1 to 3 years; (iii) one pool of four sera from condyloma accuminatum patients found reactive with a synthetic peptide derived from the L2 ORF of HPV-6b and shared by HPV-11 (Suchánková et al., 1990); (iv) one pool of three sera from INCA patients in whose biopsies HPV 18-DNA had been demonstrated by dot blot hybridization. The results are presented in Fig. 2. Peptides 16/E7-1 to 4 reacted with the first serum pool (from HPV-16 INCA patients) but not with the three other pools. Cross-reactivity was observed with the 16/E7-5 peptide, which reacted with all the serum pools except that of sera from healthy children. None of the pools reacted with peptide no. 6 to 9. These results suggested that the reactivity of peptides 1 to 4 was HPV-16-specific relative to HPV-6, -11 and -18, whereas peptide no. 5 was detected by type-common antibodies.

To test for the specificity of the reactions, adsorption experiments were carried out with one serum strongly reactive with the 16/E7-2 peptide. The results are summarized in Tables 1 and 2. It can be seen that after adsorption with the homologous peptides the reactivity with these peptides dropped to background levels. Preincubation of serum with bacterial lysate containing the HPV-16 E7 fusion protein completely removed the reaction with 16/E7-2 peptide while reducing reactivity with 16/E7-5 by 50%. Similar treatment with parental or E4 peptide-expressing bacteria did not change the ELISA reading for either the 16/E7-2 or 1/E7-5 peptide.

The results of antibody determinations in sera from CIN and INCA patients and their matched controls are summarized in Table 3. Seven out of 29 INCA sera (i.e. 24%) and two control sera reacted with the 16/E7-2 peptide (P < 0.1). Of the seven 16/E7-2 antibody-positive sera five were also reactive with 16/E7-1 and 16/E7-3 peptides and one was reactive with the 16/E7-4 peptide. The reactions with peptides 1 to 3 were much less frequent in CIN patients or control subjects. Reactivity with 16/E7-5 was much more common both among the INCA patients and their controls. Though positive reactions were somewhat more frequent in the patients than the controls, this difference was not statistically significant. It was not the purpose of the present study to test systematically the correlation between the results of hybridization tests for the presence of HPV DNA and the presence of antibody to the peptides. Still, in a number of individuals both tests were performed. The results of hybridizations are indicated in the footnote to Table 3. In spite of their suggesting that antibodies to 16/E7-1 to -3 peptides may be more frequent in subjects infected with HPV-16 than with other HPV types, it
Table 1. Adsorption of 16/E7-2-reactive antibody from a strongly reactive human serum

<table>
<thead>
<tr>
<th>Adsorbed with</th>
<th>Reactivity* with</th>
<th>1/E7-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16/E7-2</td>
<td>1/E7-1</td>
</tr>
<tr>
<td>−</td>
<td>2.583</td>
<td>0.136</td>
</tr>
<tr>
<td>16/E7-2 peptide</td>
<td>0.234</td>
<td>0.176</td>
</tr>
<tr>
<td>MS2†</td>
<td>2.167</td>
<td>0.157</td>
</tr>
<tr>
<td>MS2 E4‡</td>
<td>2.135</td>
<td>0.210</td>
</tr>
<tr>
<td>MS2 E7‡</td>
<td>0.448</td>
<td>0.243</td>
</tr>
</tbody>
</table>

* Expressed as absorbance.
† Lysate of the parental C600/537 MS2 strain of E. coli.
‡ Lysates of MS2 E4 and MS2 E7, i.e. MS2 derivatives with expression plasmids containing HPV-16 E4 and E7, respectively, prepared after induction for 3 h at 42°C (Jochmus-Kudielka et al., 1989).

Table 2. Adsorption of 16/E7-5-reactive antibody from a strongly reactive human serum

<table>
<thead>
<tr>
<th>Adsorbed with</th>
<th>Reactivity* with</th>
<th>1/E7-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16/E7-5</td>
<td>1/E7-2</td>
</tr>
<tr>
<td>−</td>
<td>2.166</td>
<td>0.330</td>
</tr>
<tr>
<td>16/E7-5 peptide</td>
<td>0.233</td>
<td>0.400</td>
</tr>
<tr>
<td>MS2†</td>
<td>2.022</td>
<td>0.367</td>
</tr>
<tr>
<td>MS2 E4‡</td>
<td>1.856</td>
<td>0.355</td>
</tr>
<tr>
<td>MS2 E7‡</td>
<td>1.080</td>
<td>0.293</td>
</tr>
</tbody>
</table>

* † ‡ For explanations see Table 1.

Discussion

The aim of our present work is to identify and prepare peptides suitable for serological study of HPV infections. Here we report on the measurement of the antibody response to synthetic peptides derived from the early (E7) protein of HPV-16. There were three main reasons for choosing the E7 protein region. First, the HPV-16 E7 protein is consistently expressed in tumour cell lines (Watanabe et al., 1989) and in biopsy-derived cells (Schwarz et al., 1985) that contain HPV-16. Second, it is the most abundant viral protein in cell lines containing HPV-16 (Smotkin & Wettstein, 1986). Third, there is a presumed difference between the biological activity of E7 in HPV types with different degrees of association with gynaecological tumours (types 16, 18, 31 and 33 are the most frequently associated, and types 6 and 11 less frequently) (Goldsborough et al., 1989). For example, cotransfection of an activated ras gene with E7 ORF of HPV types 16, 18, 31 or 33 resulted in transformation of primary epithelial cells, whereas the cotransfection of ras with the E7 gene of HPV-6 or -11 failed to produce foci of transformed cells (Storey et al., 1988). The E7 protein of

Table 3. Antibodies to peptides derived from E7 ORFs of HPV-16 and HPV-1

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of specimens</th>
<th>No. reactive (%) with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16/E7-1</td>
<td>16/E7-2</td>
</tr>
<tr>
<td>CIN I–III*</td>
<td>24</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Controls</td>
<td>24</td>
<td>0 (0)</td>
</tr>
<tr>
<td>INCA†</td>
<td>29</td>
<td>5 (17)</td>
</tr>
<tr>
<td>Controls</td>
<td>29</td>
<td>1 (3)</td>
</tr>
</tbody>
</table>

* Thirteen biopsy specimens were examined by hybridization in situ. Of these, one was positive for HPV-16 DNA and four for other HPV type DNAs. Serum from the HPV-16-positive subject was not reactive with any antigen. None of the patients positive for other HPV types possessed antibody reactive with 16/E7-1 to -4 peptides but all possessed anti-16/E7-5 antibody. Of the eight negative patients one was 16/E7-2 antibody-positive and six were 16/E7-5 antibody-positive.
† Twelve biopsy specimens were examined by dot blot and/or Southern blot hybridization. Of these, eight were positive for HPV-16 DNA and three for other HPV type DNAs. As concerns the HPV-16 DNA-positive patients, sera of three reacted with one or more of the 16/E7-1 to -4 peptides, and five of them with the 16/E7-5 antigen. None of the three patients positive for other HPV DNA types possessed anti-16/E7-1 to -4 antibody but two possessed the anti-16/E7-5 antibody. Sera from HPV-negative patients were free of antibodies against all antigens used.

seems clear that only some, possibly a minority, of HPV-16 DNA-positive patients had developed this type of antibody. The lack of any association between the presence of 16/E7-5 antibody and the outcome of the hybridization test was in line with previous results suggesting a broad-range reactivity of this antigen.
HPV-16 has also been shown to activate the enhancer for the adenovirus 5 E2 promoter (Phelps et al., 1989). Hence, the consistent expression of E7 in tumour cells, most probably conditioned by a role it plays in the maintenance of the transformation state, has stimulated a search for antibody to this protein in sera of INCA patients.

Of nine overlapping peptides that jointly cover the entire amino acid sequence of the E7 protein, four peptides derived from its N-terminal portion (amino acids 1 to 50) seemed to react specifically to type 16 whereas the fifth one derived from its central portion (41 to 60) was apparently cross-reactive. This suggestion originally derived from the investigations of defined serum pools was further corroborated by tests on individual patients' sera. Peptide 16/E7-2 provided the highest score of reactivity with sera from INCA patients; this suggests that it is this one of the peptides tested which may be the most reliable tool for HPV-16 antibody detection. The reactivity of some of the control sera from patients in whose biopsies HPV-16 DNA had not been detected does not necessarily conflict with this suggestion; the few positive sera might originate from HPV-16-infected patients. It remains to be determined whether the detection of anti-16/E7-2 antibody may help to identify individuals at increased risk of cervical cancer and possibly also those with a current but still undetected malignant process, or whether it may help in monitoring the clinical state of patients treated for HPV-16-associated genital cancer. If so, it seems clear that it could be utilized only in some patients. From the present serological data and their preliminary correlation with the hybridization data (see footnote to Table 3), it has to be inferred that only a proportion of HPV-16-infected patients develop antibodies to the type-specific reactive epitopes of the E7 protein. A similar conclusion can be drawn from the serological study of Jochmus-Kudielka et al. (1989) who used a genetically engineered E7 protein. Of their cervical cancer patients only 20% possessed the respective antibody. However, some other HPV-16-derived peptides were found to be reactive with sera from cervical cancer patients (Dillner et al., 1989). It is to be hoped that a mixture of peptides derived from a variety of HPV-16 proteins that will react in most HPV-16-infected individuals might be constituted in the not too distant future.

When amino acid sequences of the 16/E7-2 and 16/E7-5 peptides were compared with the corresponding sequences in HPV types 6, 11 and 18, paradoxically it was the 16/E7-2 peptide that showed the higher homology (Fig. 3). This is in apparent disagreement with our serological results: the 16/E7-5 peptide displaying lower homology on the amino acid sequence level with analogous peptides of other HPV types seemed to detect type-common antibodies (Table 2).

Despite the fact that the precise epitopic sites in the peptides under discussion were not identified, it may be useful to try to explain this discrepancy. The specificity of the 16/E7-2 peptide in reacting only with HPV-16-infected patients' sera can be explained in terms of conformational predisposition of peptides for antigen-antibody interaction. In the HPV-6- and HPV-11-derived analogues of the 16/E7-2 peptide there is a strong propensity to form a β-turn in residues PDVP (P = 3.74 x 10^-4). Since B cell determinants are often located around β-turns (Krčňák et al., 1989a), this conformation may play an essential role for interaction with antibodies. No such structural feature can be found in the 16/E7-2 peptide and therefore this peptide very probably adopts different conformations. As a consequence, antibodies in sera of HPV-6- and HPV-11-infected patients are not recognized by the 16/E7-2 peptide. On the other hand, the 16/E7-5 peptide and its corresponding sequences in HPV types 6, 11 and 18 show a very similar structural pattern: a high Chou-Fasman potential for β-sheet formation starting at residue 47 preceded by an α-helical segment in all four proteins. These uniform structural features are probably responsible for the reactivity of the 16/E7-5 peptide with sera of patients infected by HPV types 6, 11 or 18.

References


(Received 10 May 1990; Accepted 8 August 1990)