Epitope mapping of the human papillomavirus type 16 E4 protein by means of synthetic peptides

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Eight overlapping icosaapeptides covering the entire sequence of the E4 protein of human papillomavirus type 16 (HPV-16), were prepared and tested for their reactivity with human sera in IgG-specific ELISA. The strongest reactivity of sera from HPV-16 DNA-positive invasive cervical carcinoma (INCA) patients was detected with the peptide denoted 16/E4-6, covering amino acids 51 to 70. Subsequently nearly 200 sera were tested for the presence of the 16/E4-6-specific antibody. Reactivity was more frequent in cervical intraepithelial neoplasia patients and INCA patients than in matched control subjects. Sera from INCA patients were also tested for antibody reactive with peptide 16/E7-2 covering the major type-specific reactive region of the HPV-16 E7 protein. Only four of 13 sera possessing the 16/E4-6-specific antibody were reactive with the 16/E7-2 peptide.

The development and evaluation of type-specific serological tests for diagnosis of human papillomavirus (HPV)-related cervical cancer has recently received much attention (Dillner et al., 1989; Dillner, 1990; Jochmus-Kudielka et al., 1989; Jenison et al., 1989, 1990, 1991; Krchňák et al., 1990; Mann et al., 1990; Suchánková et al., 1990, 1991; Köchel et al., 1991). Although the majority of the research has been focused on the E7 protein, the product of the E4 open reading frame (ORF), which is poorly conserved among different HPV types (Doorbar et al., 1989), has also emerged as a possible candidate antigen in such tests. The E4 protein of HPV-1 is expressed abundantly in HPV-1-associated warts (Croissant et al., 1985; Doorbar et al., 1986) and is believed to play a role in virus maturation. Although an abundant E4-specific mRNA spliced to the 5' end of the E1 ORF was found in genital warts (Chow et al., 1987) and in a human cell line immortalized by HPV-16 DNA (Dürst et al., 1987), little is known about the extent of E4 ORF expression in genital precancerous and cancerous lesions associated with HPV-16. Distribution of anti-E4 antibodies in human sera was first reported by Jochmus-Kudielka et al. (1989) who used the HPV-16 E4 fusion protein as the antigen in Western blot (WB) assays. They have detected IgG anti-E4 antibody in 16% of invasive cervical carcinoma (INCA) patients and in 8% of matched control women; however, up to 40% anti-E4-positive sera were found among healthy teenagers and among women suffering from cervical intraepithelial neoplasia (CIN). On the basis of these data the authors concluded that the presence of E4-specific antibody correlates more closely with virus replication than with the development of cancer. Köchel et al. (1991) who also employed HPV-16 fusion proteins as antigens in WB detected anti-E4 antibody in three out of four genital carcinoma patients but not in any of the 63 healthy women tested. Using an ELISA with synthetic peptides derived from the ORF of HPV-16 E4, Dillner (1990) found the major IgG- and IgA-reactive epitope(s) in a peptide covering amino acids 47 to 66. The IgG antibody reactive with this peptide was detected in 37% of INCA patients and in 28% of control subjects; the prevalence of the IgA antibody was 67 and 33%, respectively, suggesting that the positions of the immunoreactive epitopes can differ for different Ig classes. To identify seroreactive regions of the HPV-16 E4 protein, Müller et al. (1990) used HPV-16 E4 antigens expressed as part of the capsid protein of genetically modified phage fd and synthetic octapeptides derived from the HPV E4 ORF. Employing rabbit sera prepared by immunization with the HPV-16 E4 fusion protein they located the major immunoreactive region between amino acids 34 and 47.

Recently we have reported the mapping of HPV-16 E7 epitopes reactive with human sera using a series of overlapping peptides covering the entire sequence of this protein (Krchňák et al., 1990; Suchánková et al., 1991). In the present experiments we adopted a similar approach to delineate regions of the HPV-16 E4 ORF-encoded protein containing immunoreactive epitopes.

Sera used in the present study originated from both
Fig. 1. Reactivity (A600) in ELISA of pooled sera with individual peptides derived from HPV-16 E4 ORF. , HPV-18 DNA-positive; , HPV-16 DNA-positive; , HPV-16 E4 WB-positive; , HPV-16 E4 WB-negative; , children, 1 to 3 years.

healthy women and those suffering from either CIN or INCA. The group of healthy women consisted of 87 subjects aged 19 to 49 years who had been investigated at the Center of Cervical Cancer Prevention, Third Medical Faculty, Charles University, Prague, and found to be free of any pathological colposcopical and cytological lesions. Fifteen sera originated from women with CIN (I to III) aged 24 to 50 years and 30 sera from women suffering from INCA aged 33 to 79 years. Biopsy specimens for hybridization tests (Krchňák et al., 1990) were available from 12 INCA patients. HPV-16 DNA was demonstrated in six and HPV-18 DNA in three specimens. Control groups of healthy women matched by age and the area in which they lived were constituted for each group of patients; these subjects were not investigated gynaecologically. In addition, sera from 30 children aged 1 to 11 years were also included. Eight icosapeptides consecutively overlapping each other by 10 amino acids and covering the entire sequence of the E4 protein were prepared by continuous-flow solid-phase multiple peptide synthesis and were purified as described previously (Krchňák et al., 1990). An ELISA for detecting IgG antibodies was performed as described (Krchňák et al., 1990). The HPV-16 E4 fusion protein and WB technique were the same as those used by Jochmus-Kudielka et al. (1989).

For screening tests we used 2 μg of each peptide per well and five serum pools each containing at least three sera. Pool no. 1 originated from INCA patients in whose biopsies HPV-18 DNA was detected. Pool no. 2 was formed by sera from HPV-16 DNA-positive INCA patients which possessed antibody reactive with HPV-16 E4 in WB. Another pool (no. 3) was formed by sera from INCA patients whose sera were HPV-16 E4 antibody-positive in WB but were not tested for the presence of HPV DNA. Pool no. 4 consisted of INCA patients' sera which were free of HPV-16 E4 antibody in WB and were not tested for the presence of HPV DNA. Finally, pool no. 5 was created by mixing sera from healthy children. The ELISA results with the eight peptides are shown in Fig. 1. The strongest reactivity occurred for peptide 16/E4-6 (amino acids 51 to 70) with the two serum pools (nos. 2 and 3) originating from INCA patients, positive in WB for the HPV-16 E4 antibody. Sera which were free of the E4 antibody in WB assay, originating from HPV-18 DNA-positive patients and from other INCA patients, were non-reactive with this peptide. Some reactivity, although of lower intensity, was also observed with peptides 1 to 5. However, due to the relatively high reactivity of control children's sera, the specificity of the reactions with peptides 16/E4-1, 16/E4-2 and 16/E4-5 is questionable. None of the serum pools was reactive with peptides corresponding to the C-terminal portion of the E4 protein.

For subsequent tests only the 16/E4-6 peptide was used. In all the tests 4 antigen units of the 16/E4-6 peptide were used, 1 antigen unit being defined as the highest antigen dilution reactive with a 1:20 dilution of serum pool no. 3. All sera were tested in a 1:20 dilution. To assign a cutoff value we tested sera from the 30 control children which we assumed had not experienced HPV-16 infection. The cutoff value selected was the mean ELISA absorbance value of these sera plus 3 S.D. (x = 0.395; s.d. 0.231; cutoff point 1.08). The icosapeptide derived from the HPV-1 E7 ORF (amino acids 11 to 30) served as control antigen; only one of nearly 200 sera tested was found reactive with this antigen.

Results of 16/E4-6 antibody determinations are summarized in Table 1. Nineteen out of 87 (i.e. 21.8%) women with normal colposcopical and cytological findings possessed the antibody. As indicated in the footnote to Table 1 within this group the prevalence of

<table>
<thead>
<tr>
<th>Group</th>
<th>No. tested</th>
<th>No. reactive</th>
<th>Percentage reactive</th>
</tr>
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<tr>
<td>Healthy subjects*</td>
<td>87</td>
<td>19</td>
<td>21.8</td>
</tr>
<tr>
<td>CIN patients</td>
<td>25</td>
<td>8</td>
<td>32.0</td>
</tr>
<tr>
<td>CIN controls</td>
<td>25</td>
<td>4</td>
<td>16.0</td>
</tr>
<tr>
<td>INCA patients†</td>
<td>30</td>
<td>13</td>
<td>43.3</td>
</tr>
<tr>
<td>INCA controls</td>
<td>30</td>
<td>4</td>
<td>13.3</td>
</tr>
</tbody>
</table>

* Prevalence of antibody in age groups < 25, 25 to 35, > 35 years was 29.1, 17.1 and 21.4%, respectively.
† Hybridization tests were performed with materials from 12 INCA patients; HPV-16 DNA was detected in six and HPV-18 DNA in three patients.
the 16/E4-6 antibody was not clearly age-dependent. The antibody was detected in eight of 25 CIN patients (32\%), but only in four of 25 (16\%) matched control women. However this difference was not statistically significant (P > 0.05). The prevalence of 16/E4-6 antibody among the INCA patients was even higher; it was detected in 13 of 30 patients (i.e. 43.3\%); among the matched control subjects it was found only in four instances (13.3\%). This difference was statistically significant (P < 0.01). Of the six INCA patients in whose biopsies HPV-16 DNA had been demonstrated, three possessed the 16/E4-6 antibody, which was also detected in sera of all three HPV-18 DNA-positive patients; it may be of interest, however, that the absorbance values of these three sera were the lowest among the sera scored as positive. These three sera were also weakly positive in the WB assay using the HPV-16 E4 protein as antigen (not shown). Sera from the three HPV DNA-negative patients were free of the 16/E4-6 antibody.

Sera from INCA patients were also tested for the presence of antibody to the 16/E7-2 peptide (corresponding to amino acids 11 to 30 of the E7 protein); this peptide has been found to cover the major type-specific reactive region of the HPV-16 E7 protein (Krchhák et al., 1990; Suchánková et al., 1991; Jenison et al., 1991).

As indicated in Table 2, of the 13 16/E4-6-positive sera only four possessed the 16/E7-2 antibody. Thirteen sera were negative in both tests. Thus the overall concordance was 56.7\%, showing a rather low degree of correlation.

The results presented here indicate that the major immunoreactive region of the HPV-16 E4 protein is located between amino acids 51 and 70. This finding is consistent with the previous observation by Dillner et al. (1989). The immunoreactive region recognized is different from that reported by Müller et al. (1990), which indicates that the antibody raised by immunizing with bacterial fusion protein was reactive with epitopes distinct from those recognized by human antibody. The prevalence of the 16/E4-6 antibody was higher than that of the 16/E7-2 antibody which had been quite rare among healthy subjects and women with CIN (Suchánková et al., 1991). However, there seems to be an association between cervical neoplasia and the presence of the 16/E4-6 antibody. Prevalence of this antibody was higher among the CIN and INCA patients than among matched control women; this does not necessarily imply that the occurrence of the 16/E4-6 antibody was associated with the development of cancer. It is possible that the difference in antibody prevalence simply reflected a higher proportion of HPV-16-infected subjects among INCA patients compared to healthy women. It is noteworthy that the antibody response to 16/E4-6 did not predict the reactivity with 16/E7-2, and vice versa. It should be recalled that Mann et al. (1990) also reported poor correlation between the presence of HPV-16 E7 antibody and antibody to peptide 245, a nonadecapeptide derived from a region close to the carboxyl terminus of the E2 protein of HPV-16 (Dillner et al., 1989). It is not clear whether the present observation was due to the variation in the response of HPV-16-infected subjects to different epitopes. Such variation may be dependent on individual sensitivities to different viral antigens as reported recently by Jenison et al. (1991) for HPV-16 and HPV-18 L2 proteins, or be a consequence of specific features of the virus–host interactions, such as the rate of virus replication or the extent of the viral expression in tumour tissue. Provided that both reactions are HPV-16-specific, the simultaneous use of the two antigens could markedly increase the capability of monitoring HPV-16 infections and/or HPV-16-associated cervical cancer by serological means. It is also possible, however, that the discrepancy reported here and that observed by Mann et al. (1990) were associated with different strain specificities of the respective antigens and that a considerable proportion of the positive reactions was due to fortuitous cross-reactions. The present observation of the positive albeit weak reactivity of three sera from HPV-18 DNA-positive patients may favour this possibility. More information and more rigorous analyses of the reactivities monitored are needed to clarify the significance of the association of the various antibodies with HPV-16 infection and, especially, with HPV-16-linked neoplasia.

**References**


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