Comparison of ELISA and Western blotting for human papillomavirus type 16 E7 antibody determination

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A total of 140 sera originating from healthy women and women with either cervical intraepithelial neoplasia or cervical cancer were tested for the presence of IgG antibody against E7 of human papillomavirus type 16 (HPV-16) by ELISA using a synthetic icosapeptide, denoted 16/E7-2, representing amino acids 11 to 30, and by Western blotting (WB) using a genetically engineered HPV-16 E7 fusion protein. Eighteen sera were found positive in either one or the other test. Positive reactions were more frequently detected in cervical carcinoma patients (12 of 34, 35.3%) than in the other individuals (six of 106, 5.7%). Ten children's (1 to 3 years of age) sera reacted in neither ELISA nor WB with HPV-16 E7. A high degree of concordance between the two tests was found suggesting that both tests detect the same or similar activity. To locate the reacting epitopes in the E7 protein, absorption tests were performed with peptides corresponding to various sections of the protein. Based on the results obtained, sera possessing antibody to HPV-16 E7 could be differentiated into those reactive with only the 16/E7-2 peptide and those reactive with other HPV-16 E7 epitopes.

Diagnosis of infection with human papillomaviruses (HPVs) suggested by colposcopic, cytological or histological findings still rests mainly on the demonstration of HPV DNA presence. In the past few years increasing efforts have been made to develop serological tests capable of proving HPV infection. In these tests, three sources of antigens have been utilized: (i) intact or disrupted HPV virions extracted either from HPV-induced lesions (Pfister & zur Hausen, 1978; Baird, 1983; Dillner et al., 1989a; Steele & Gallimore, 1990; Anisimová et al., 1990) or from virus-infected xenografts in nude mice (Christensen & Kreider, 1990), (ii) genetically engineered HPV proteins, usually in the form of bacterial fusion proteins (Jenison et al., 1988, 1989; Jochmus-Kudielka et al., 1989) or expressed as part of a capsid protein of genetically modified phage fd (Müller et al., 1990) and (iii) synthetic peptides derived from known sequences of different HPV DNA open reading frames (ORFs) (Cason et al., 1989; Dillner, 1990; Dillner et al., 1989b, 1990; Sucháneková et al., 1990; Müller et al., 1990).

Recently we developed an ELISA to detect type-specific antibody to HPV type 16 E7 protein in human sera (Krchňák et al., 1990). We examined a set of nine overlapping (by 10 amino acids), synthetic peptides covering the whole amino acid sequence of the HPV-16 E7 protein; of these eight were icosapeptides and one was an octadecapeptide. From this study it was concluded that peptides 16/E7-1 (amino acids 1 to 20), 16/E7-2 (11 to 30), 16/E7-3 (21 to 40) and 16/E7-4 (31 to 50) were type-specific whereas the 16/E7-5 peptide was broadly cross-reactive. In sera from invasive carcinoma patients, positive HPV-16-specific reactions were more frequent with the 16/E7-2 peptide than with the other peptides. This reactivity could be removed by absorption with the 16/E7-2 peptide as well as by the genetically engineered HPV-16 E7 fusion protein but not with the HPV-16 E4 fusion protein.

In the present series of experiments we tried to correlate the results obtained in ELISA, using the 16/E7-2 peptide, and by Western blotting (WB) using the HPV-16 E7 fusion protein. A total of 140 serum samples were examined. All of them were collected at the Center of Cervical Cancer Prevention, Third Medical Faculty, Charles University, Prague, from women aged 25 to 53 years. Of these, 85 were free from pathological, colposcopic and cytological findings, 21 suffered from various stages of cervical intraepithelial neoplasia (CIN I to CIN III) and 34 from invasive cervical carcinoma (INCA). Sera from 10 children aged 1 to 3 years were also
tested in parallel. All sera were inactivated by heating at 56°C for 30 min and kept at -20°C until investigated. The peptides were prepared and IgG-specific ELISA tested in parallel. All sera were inactivated by heating at 56°C for 30 min and kept at -20°C until investigated. Denatured 1/E7-1 (amino acids 11 to 30) and 1/E7-2 (amino acids 35 to 54) served as control antigens. Control sera known to be positive or negative for the HPV-16 E7 antibody in ELISA (Krchňák et al., 1990) were included in each test. All sera were tested in a 1:10 dilution. The cutoff value between positivity and negativity was the mean absorbance (A) value plus 3 S.D. of the previously tested group of sera from 44 children aged 1 to 11 years which were considered anti-HPV-16 antibody-negative (Krchňák et al., 1990).

In WB experiments, lysates of genetically engineered Escherichia coli expressing HPV-16 E7 and HPV-16 E4 fusion proteins were used (Jochmus-Kudielka et al., 1989). They were derived from the expression vectors pEX 8 mer-HPV-16 E7 and pEX 12 mer-HPV-16 E4 constructed by Seedorf et al. (1987). They contain the whole HPV-16 E7 ORF [nucleotides (nt) 585 to 855] and HPV-16 E4 ORF (nt 3399 to 3617), respectively, fused to the DNA sequence encoding the first 100 amino acids of the bacteriophage MS2 polymerase. Preparation of the expressed fusion protein from bacterial extracts and WB analysis of sera which had been preabsorbed with lysates of the parental bacteria, were performed according to Jochmus-Kudielka et al. (1989); for visualization of the antigen–antibody reaction peroxidase-labelled pig anti-human IgG (SwahHu, Sevac) was used. As a control antigen a protein comprising the first 100 amino acids of the MS2 polymerase was employed (Jochmus-Kudielka et al., 1989). For the WB assay, sera were diluted 1:20. A positively reacting serum (diluted 1:1000) was included as a positive control in each test. If not indicated otherwise each serum sample was tested twice and was scored as strongly (+ + +), intermediately (+ +) and weakly (+) positive or negative (−). With very few exceptions mentioned below, the results of the repeated tests were identical.

The results are summarized in Tables 1 and 2. Of 106 sera from healthy women and women with CIN, only five (4.7%) were reactive with 16/E7-2 peptide and by WB using the genetically engineered HPV-16 E7 fusion protein.

### Table 1. HPV-16 E7 antibody presence in sera from women without pathological, colposcopic or cytological findings and from women with CIN, as detected by ELISA using the 16/E7-2 peptide and by WB using the genetically engineered HPV-16 E7 fusion protein

<table>
<thead>
<tr>
<th></th>
<th>ELISA</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>WB+</td>
<td>3*</td>
<td>1†</td>
</tr>
<tr>
<td>WB−</td>
<td>2‡</td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>101‡</td>
</tr>
</tbody>
</table>

* A values in ELISA: 0.958, 1.438 and 2.215.
† A value 0.272.
‡ A values 0.992 and 0.820.
§ Mean A values 0.384; S.D., 0.118.
¶ Twenty-one sera originated from CIN patients; one of these was positive in both ELISA and WB, the remaining sera were negative in both tests.

### Table 2. HPV-16 E7 antibody presence in sera from patients with cervical cancer, as detected by ELISA using the 16/E7-2 peptide and by WB using the genetically engineered HPV-16 E7 fusion protein

<table>
<thead>
<tr>
<th></th>
<th>ELISA</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>WB+</td>
<td>9†</td>
<td>2‡</td>
</tr>
<tr>
<td>WB−</td>
<td>1§</td>
<td>22</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>24‡</td>
</tr>
</tbody>
</table>

* Biopsy specimens from 19 patients were examined by dot blot and/or Southern blot hybridization; 10 were positive for HPV-16 DNA and three for HPV-18 DNA. Within the group of HPV-16 DNA-positive patients, four sera were reactive in both ELISA and WB whereas the others were non-reactive in either test. None of the three HPV-18 DNA-positive patients possessed antibody reactive in ELISA but two possessed antibody reactive in WB.
† A values in ELISA: 2.412, 2.368, 1.674, 2.490, 0.900, 1.147, 0.916, 1.694 and 1.935.
‡ A values 0.217 and 0.144.
§ A value 1.565.
¶ Mean A value 0.262; S.D., 0.104.

ELISA and WB was similar to those in the previous group of patients (Table 2). Ten (29.4%) were reactive with 16/E7-2 in ELISA and of these nine were also positive in WB with HPV-16 E7. Two sera non-reactive in ELISA were clearly positive in WB. Again concordant results were obtained in a great majority of sera (kappa value 0.7935 ± 0.2896, Student's t-test 2.740, P < 0.01). Taken together, of 15 sera positive in ELISA, 12 (80%) were also reactive in WB; of 125 sera non-reactive in
ELISA 97.6\% were also negative in WB. Overall concordance of results was 95.7\% (kappa 0.776 ± 0.190, Student's t-test 4.083, P < 0.001). In addition, as indicated in Table 3, in most sera there was a good correlation between the strength of the reactions in the two tests. None of the 10 control children's sera was reactive in either ELISA or WB with HPV-16 E7. Only one of the sera positive in ELISA and/or WB with HPV-16 E7 antigens was reactive in ELISA with the control MS2 polymerase antigen.

The data presented in Tables 1 and 2 indicate a high degree of concordance between the two tests suggesting that both tests are monitoring the same or similar activity and that the major immunoreactive region of the HPV-16 E7 protein is covered by the 16/E7-2 peptide. Three sera were positive in WB and negative in ELISA. These sera did not possess antibodies to linear epitopes positioned outside 16/E7-2 as none of them reacted in ELISA with any of the nine overlapping peptides covering the whole amino acid sequence of the HPV-16 E7 protein (not shown). This suggested that epitopes present in the complete protein sequence and not in the synthetic peptides corresponding to parts of the complete sequence were involved. The presence of non-linear epitopes has recently been suggested by the use of a panel of monoclonal antibodies (MAbs) raised against the HPV-16 E7 fusion protein and it has been shown that the respective MAbs were cross-reactive with the HPV-18 E6 protein in both ELISA and WB (Tindle et al., 1990). In this respect it is of interest that two of the discordant sera originated from INCA patients whose biopsy materials contained HPV-18 DNA (not shown). Thus, the absence of reactivity in ELISA might be due to the fact that these patients did not experience HPV-16 infection and the positive signal in WB could be due to cross-reacting antibodies. The opposite findings, i.e. positivity in ELISA and negativity in WB, were obtained in three sera. The discrepancy in two of them was apparently associated with their borderline activity; in fact, in repeated ELISA their A values with 16/E7-2 were either just below or just above the cutoff point. Similarly, in one of the three repeated WB assays both sera gave a weakly positive signal. On the other hand, the third serum originating from an INCA patient exhibited reproducibly the WB- and ELISA-positive pattern. The nature of this discrepancy is not understood.

To obtain more information about the location of the reacting epitopes within the HPV-16 E7 protein, absorption experiments were carried out. Seven serum samples strongly reactive in both tests were selected. They differed in their pattern of reactivity in ELISA (Krchňák et al., 1990). Sera nos. 1 and 2 were reactive only with the 16/E7-2 peptide (A values of 1.438 and 2.215, respectively), whereas the other three sera exhibited a broader reactivity. A values for serum no. 3 with 16/E7-2 and 16/E7-3 were 1.147 and 1.087, respectively. A values for serum no. 4 with 16/E7-1, 16/E7-2 and 16/E7-3 were 0.875, 1.674 and 2.507, respectively. The corresponding values for serum no. 5 were 1.373, 2.490 and 1.398, respectively. In absorption experiments the peptides were used either individually or in mixtures. In addition to those already mentioned (see above), the following peptides were also used: 16/E7-5 (amino acids 41 to 60), 16/E7-6 (51 to 70), 16/E7-7 (61 to 80), 16/E7-8 (71 to 90) and 16/E7-9 (81 to 98). The serum–peptide mixtures were incubated for 1 h at 37 °C and overnight at 4 °C and centrifuged for 10 min at 4000 r.p.m. The supernatants were tested by WB for the presence of antibody. Results obtained with serum no. 1 are shown in Fig. 1. It can be seen that after absorption with the 16/E7-2 peptide alone the reactivity in WB of this serum still remained positive. Treatment with any of the HPV-16 E7 peptides. As expected, the absorbed serum lost its reactivity with the 16/E7-2 peptide in ELISA. Similar results were obtained with serum no. 2 (not shown). Results obtained with serum no. 3 are shown in Fig. 2. Its reactivity clearly differed from that of sera nos. 1 and 2. Although absorption with 60 μg of the 16/E7-2 peptide reduced the WB reactivity markedly, it did not remove it completely; however, treatment with a mixture of 16/E7-1, 16/E7-2 and 16/E7-3 peptides resulted in nearly complete removal of the WB reactivity. After absorption with the

Table 3. Correlation between reactivity in ELISA and intensity of bands in WB of ELISA- and/or WB-positive sera

<table>
<thead>
<tr>
<th>WB intensity*</th>
<th>0.316†</th>
<th>0.317–1.500</th>
<th>&gt;1.500</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>−</td>
<td>−</td>
<td>1</td>
</tr>
<tr>
<td>+</td>
<td>2‡</td>
<td>2</td>
<td>−</td>
</tr>
<tr>
<td>++</td>
<td>1</td>
<td>3</td>
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</tr>
<tr>
<td>+++</td>
<td>−</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>6</td>
<td>9</td>
</tr>
</tbody>
</table>

* −, Negative; +, weakly positive; ++, intermediate positive; ++++, strongly positive.
† Cutoff value.
‡ These three sera were scored in repeated tests as negative or weakly positive.
found a single major immunoreactive region in the HPV-16 E7 protein between amino acids 21 and 34. This region is contained within our peptides 16/E7-2 and 16/E7-3. From the data presented here it appears that more than one epitope may be present in this region. Also, other epitopes may be present in the N-terminal part of the E7 protein as reported by Tindle et al. (1990), Dillner (1990) and Müller et al. (1990) and as suggested here.

In conclusion, the present data seem to indicate that sera possessing the anti-HPV-16 E7 antibody detectable by both ELISA and WB can be divided according to their reactivity into several groups, i.e. those possessing antibodies to epitope(s) fully contained within the 16/E7-2 peptide, and those possessing, in addition, antibodies to epitopes outside the area covered by the 16/E7-2 peptide. The latter can possibly be further divided into those predominantly possessing antibodies to continuous epitopes localized within the N-terminal half of the peptide and those possessing antibodies to more widely distributed continuous epitopes or to discontinuous conformational epitopes present in the HPV-16 E7 protein.

16/E7-2 peptide the reactivity in ELISA with this peptide was completely removed; it remained unchanged by absorption with 16/E7-1 and 16/E7-5 peptides (not shown). Results obtained with sera nos. 4 and 5 using the same peptides were different; none of the treatments resulted in substantial reduction of reactivity in WB (not shown).

There appears to be an agreement of our previous (Krchňák et al., 1990) and present data with those presented recently by Jenison et al. (1991). Using nested sets of deleted recombinant proteins these authors have

References


Short communication


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