*Acta virol.* 37: 395–402, 1993

# IDENTIFICATION OF SEROREACTIVE EPITOPES OF HUMAN PAPILLOMAVIRUS TYPE 18 E7 PROTEIN BY SYNTHETIC PEPTIDES

V. KRCHŇÁK[1], T. PIŠTĚK[1], J. VÁGNER[1], A. SUCHÁNKOVÁ[2], J. KAŇKA[3], L. RITTEROVÁ[4], V. VONKA[4]*

[1]Research Institute for Feed Supplements and Veterinary Drugs, Jílové near Prague; [2]Institute of Sera and Vaccines, Prague; [3]Department of Obstetrics and Gynaecology, Third Medical Faculty, Charles University, Prague; and [4]Department of Experimental Virology, Institute of Haematology and Blood Transfusion, Korunní 108, 101 03 Prague, Czech Republic

*Received December 17, 1992*

*Summary.* – Nine everlapping peptides covering the entire sequence of early protein E7 of human papillomavirus type 18 (HPV-18) were synthesized and tested as antigens with pools of selected human sera in ELISA. Peptides denoted 18/E7-2, 18/E7-3, and 18/E7-5 (amino acid positions 11–33, 21–40, and 41–60, respectively) were reactive with pooled sera originating from HPV-18 DNA-positive cervical cancer patients but not with sera from HPV-16 DNA-positive cervical cancer patients or from condyloma acuminata patients. This suggested that the epitopes contained in these peptides were HPV-18 type-specific, relative to HPV types 16, 11, and 6. On the other hand, 18/E7-1 (aa 1–23) and 18/E7-6 (aa 51–70) peptides were cross-reactive. The prevalence of antibodies reactive with 18/E7-2, 18/E7-3, and 18/E7-5 peptides in cervical carcinoma patients was very low. Thus, the utilization of these peptides for monitoring HPV-18 infection seems to be rather limited.

*Key words: synthetic peptides; ELISA; human papillomavirus*

## *Introduction*

HPVs are a widespread family of viruses that cause various proliferative changes of in the infected epithelium (zur Hausen, 1989). To date more than 70 types of HPVs have been identified and more than dozen types have been shown to be associated with genital tract neoplasms. Recent investigations have pointed out that HPV-18 may be a particularly high-risk virus. HPV-18 associated cancers occurred a decade earlier than those associated with other HPV types, and HPV-18 has also been associated with higher-grade tumours (Barnes *et al.*, 1988) and increased frequency of recurrence (Walker *et al.*, 1989).

*Corresponding author

At present, only limited evidence on the immune responses to HPV-18 proteins is available. Jenison *et al.* (1990) did not find any difference in reactivity of HPV-18 E7 recombinant protein with sera of patients with venereally transmitted diseases and of controls. Bleul *et al.* (1991) studied seroreactive regions within HPV-18 E7 and E6 proteins by immunoscreening of bacteriophage expression library and by overlapping decapeptides covalently bound to polyethylene pins. Based on mapping experiments, HPV-18 E7 - derived 28-peptide (aa 32–59) was synthesized and tested with sera of invasive cervical carcinoma (INCA) patients from Tanzania and Germany. A higher prevalence of E7 antibodies was found in the Tanzanian group of INCA sera (10 out of 110 were positive) when compared with the latter group (3 out of 94 were positive). Köchel *et al.* (1991) expressed HPV-18 gene products in *E. coli* as beta-gal fusion proteins and employed these proteins in Western blot analysis to identify HPV-18 immunoreactive regions. Out of 46 INCA patients 7 were reactive with HPV-18 E7 bacterial fusion protein, whereas none of 46 controls reacted.

We have already reported on mapping of linear epitopes of HPV-16 E7 (Krchňák *et al.*, 1990) and E4 (Suchánková *et al.*, 1992) proteins using synthetic peptides. In this communication we identified seroreactive regions within HPV-18 E7 protein and tried to find out which of them contained type-specific epitopes.

## *Materials and Methods*

*Preparation of synthetic peptides.* Peptides were prepared by continuous-flow solid-phase multiple peptide synthesis (Krchňák and Vágner, 1990) on p-methyl-benzhydrylamine resin using Fmoc/t-Bu protection strategy. Fmoc groups were removed by piperidine, condensation was performed by HOBt esters, and coupling reaction was monitored by bromophenol blue (Krchňák *et al.*, 1991). 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 correct amino acid composition and showed the expected molecular peak in fast atom bombardment mass spectroscopy.

*ELISA.* Wells of plastic plates (Nunc, Denmark) were coated with peptides (2 $\mu$g) dissolved in 10 mmol/l sodium carbonate-bicarbonate buffer, pH 9.5. The plates were incubated at 37 °C for 2 hrs and at 4 °C overnight. Unoccupied sites were blocked with 1 % bovine serum albumin (BSA) in sodium carbonate-bicarbonate buffer, pH 9.5, for 1 hr at room temperature and the plates were washed five times with PBS-Triton X-100 (0.1 %), pH 7.2 (washing solution). Serum samples diluted 1:20, unless otherwise stated, in PBS-Triton X-100, pH 7.2, with 10 % bovine serum were added and incubated for 1 hr at room temperature. After washing five times with the washing solution, 100 $\mu$l of peroxidase-labelled swine anti-human IgG (Sevac, diluted 1:2000) was added and incubated for 1 hr. The plates were then washed five times with washing buffer and 100 $\mu$l of substrate (0.04 % orthophenylenediamine, Sigma) was added in 50 mmol/l phosphate-citrate buffer (pH 5.0). The reaction was stopped by adding 100 $\mu$l 2 mol/l sulphuric acid and the absorbancy was measured at 490 nm using a Dynatech Microplate Reader.

*Sera.* For screening purpose we prepared four serum pools consisting of at least three individual serum samples: (a) pool of sera from INCA patients in whose biopsies HPV-18 DNA has been detected by dot blot hybridization (Krchňák *et al.*, 1990); (b) pool of sera from INCA patients in whose biopsies HPV-16 DNA has been detected and found to be reactive with HPV-16-derived antigens (Krchňák *et al.*, 1990; Suchánková *et al.*, 1992); (c) pool of sera from condyloma acuminata patients found reactive with synthetic peptide corresponding to epitope common to HPV-6b and

HPV-11 L2 ORF (Suchánková *et al.*, 1990); (d) pool of sera obtained from healthy children aged 1–2 years.

The following individual sera were tested: 30 sera from healthy children aged 1–3 and 9–11 years (these sera had been collected during various vaccination studies and served for setting the cut-off value), the second group of 65 sera originated from INCA patients and the third one from 65 healthy control subjects matched with the patients by age and area of living. None of these sera was included in the above mentioned pools.

The sera were inactivated by heating at 56 °C for 30 mins and kept at -20 °C until investigated. All sera were tested in 1:20 dilution and cut-off value between positivity and negativity was set at the mean absorbance plus 3 S. D. of a group of 30 sera obtained from children aged 1–11 years, which were considered anti-HPV-18-antibody negative. Control sera known to be positive or negative for HPV-18 E7 antibody were included in each ELISA test. Eicosapeptide 1/E7-1 derived from HPV-1 E7 ORF (aa 11–30) served as control antigen.

*Competition assays.* Absorption of the reactive antibody in human serum was done with the control antigen (peptides 1/E7-1) and peptide 18/E7-2 according to the procedure described in our previous communication (Krchňák *et al.*, 1990).

## *Results*

Amino acid sequences of the nine overlapping peptides comprising the whole HPV-18 E7 protein and consecutively overlapping each other by 10 amino acids

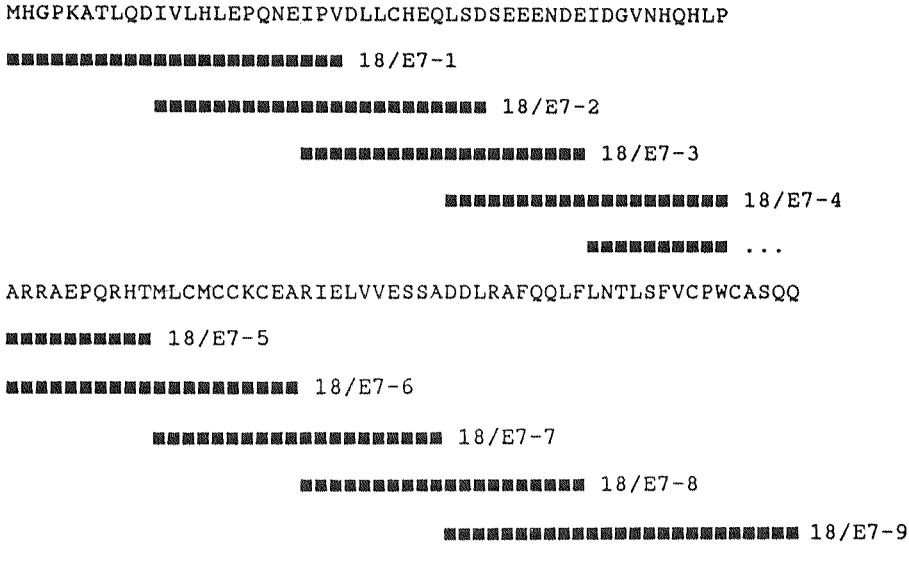


**Fig. 1**
Amino acid sequence of HPV-18 E7 protein and location of synthetic peptides

are shown in Fig. 1. Synthetic peptides were tested with four serum pools as antigens in ELISA to detect immunoreactive epitopes. The results of ELISA tests are given in Fig. 2. None of the pools was reactive with peptides 18/E7-7, 18/E7-8, and 18/E7-9. Peptides 18/E7-2, 18/E7-3, and 18/E7-5 reacted with the first serum pool (HPV-18 DNA-positive INCA patients) only. Cross-reactivity with HPV-16- and HPV-6/11-reactive sera was observed with peptides 18/E7-1 and 18/E7-6. This suggested that peptides 18/E7-2, 18/E7-3, and 18/E7-5 were HPV-18-type specific relative to HPV-16, HPV-6, and HPV-11, whereas peptides 18/E7-1 and 18/E7-6 were cross-reactive.

On the basis of the reactivity pattern with pooled sera we selected peptides 18/E7-2, 18/E7-3, and 18/E7-5 and a control eicosapeptide derived from HPV-1 E7 ORF (denoted 1/E7-1, aa 11–30) as antigens in ELISA for further study. Results of antibody determination in selected groups of sera are summarized in Table 1.

Among the INCA patients, one possessed antibodies in her serum to peptides 18/E7-2, 18/E7-3, and 18/E7-5 and another one possessed antibody against 18/E7-5 antigen only. One of these sera originated from one of the four patients with the HPV-18 DNA-positive biopsies. Antibody reactivity against peptides 18/E7-2, 18/E7-3, and 18/E7-5 was not detected in any serum sample of the control group.

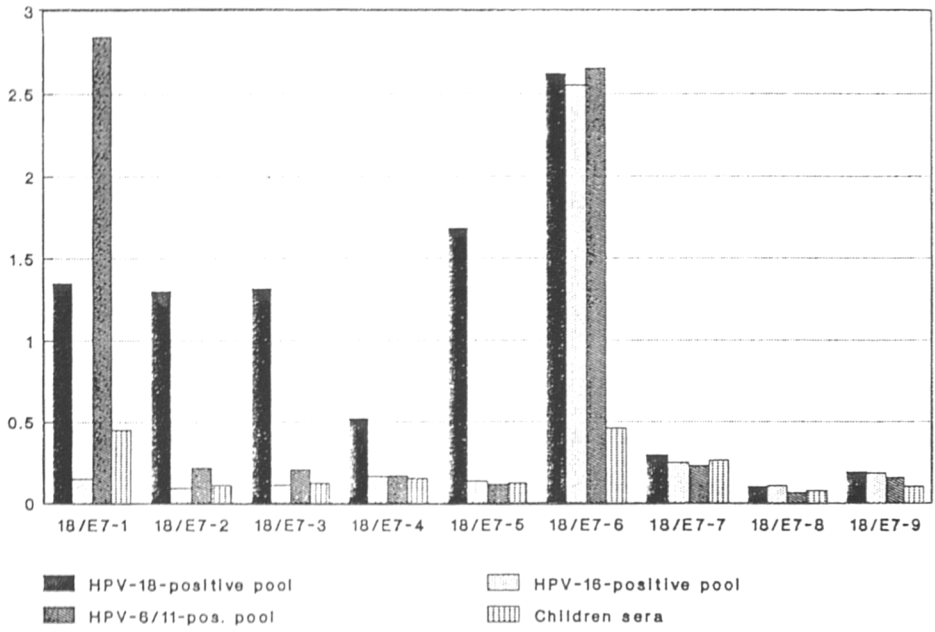


**Fig. 2**
Reactivity of pooled sera with individual HPV-18 E7 derived peptides

**Table 1. HPV-18 E7 antibody presence in sera of invasive carcinoma patients and matched control subjects**

| Group | No. tested | No. reactive with | | | |
|---|---|---|---|---|---|
| | | 18/E7-2 | 18/E7-3 | 18/E7-5 | 1/E7-1[b] |
| INCA patients[a] | 65 | 1 | 1 | 2 | 0 |
| Control subjects | 65 | 0 | 0 | 0 | 0 |

[a]Biotopies from 16 patients were tested for the presence of HPV DNA by dot blot hybridization (Krchňák *et al.*, 1990). Of these, 6 were positive for HPV-16 DNA and 4 for HPV-18 DNA. Within the group of HPV-16 DNA-positive patients all sera were negative. Serum of 1 of the 4 HPV-18 DNA-positive patients was reactive with HPV-18 E7 derived peptides.
[b]Eicosapeptide derived from HPV-1 E7 ORF (aa 11-30) served as control antigen.

The specificity of the reaction was confirmed by competition assay. The absorption of peptide 18/E7-2-reactive antibodies occurred after preincubation with 18/E7-2 peptide as evidenced by decreased $A_{490}$ values in ELISA. A similar treatment with 1/E7-1 was without effect (results not shown).

## *Discussion*

HPV types 6 and 11 are most often found in benign condylomas, while HPV types 16 and 18 (and also 31, 33, and 35) are frequently detected in invasive carcinomas. The establishing of type of HPV involved already at the early stage of the cervical pathological process (detected by cytology and/or colposcopy) may provide important leads for the treatment and subsequent follow-up of the disease. HPV proteins do not represent suitable antigens for serological screening due to the antibody cross-reactivity caused by a high degree of homology on the amino acid sequence level in different HPV types. Synthetic peptides, however, have already been shown to be capable of detecting type-specific reaction with sera obtained from patients infected with various HPV types (Müller *et al.*, 1990; Krchňák *et al.*, 1990; Dillner, 1990; Bleul *et al.*, 1991; Müller *et al.*, 1990).

The distribution of immunoreactive epitopes on HPV-18 E7 protein showed a similar pattern when compared with the location of epitopes on E7 protein of type 16, recently described by us (Krchňák *et al.*, 1990). Carboxyterminal part of both proteins did not contain any seroreactive epitopes, while aminoterminal parts contained epitopes reactive with type-specific antibodies. These results seem to be in line with those reported by Selvey *et al.* (1990) and Tindle *et al.* (1990) who used panels of monoclonal antibodies raised against genetically

```
    5     0     5     0     5     0     5     0     5     0
MHGDTPTLHEYMLDLQPETT    DLYCYEQLNDSSEEEDEIDG PAGQAEP      HPV 16 E7
***   **     * * *       ** * *** ** ** *****   *  *
MHGPKATLQDIVLHLEPQNEIPVDLLCHEQLSDSEEENDEIDGVNHQHLP      HPV 18 E7
***   ** **** *  *   ** * * *** ** *  ** * *  *
MHGRHVTLKDIVLDL  QPPDPVGLHCYEQLVDSSE  DEVDEVDGQDSQ      HPV 6b E7
```

```
    5     0     5     0     5     0     5     0     5     0     5
DR AHYNIVTF   CCKCDSTLRLCVQSTHVDIRTLEDLLMGTLGIVCPICSQKP HPV 16 E7
 * *     *    ****  * * *   * *    *   **  *** *    *
ARRAEPQRHTMLCMCCKCEARIELVVESSADDLRAFQQLFLNTLSFVCPWCASQQ HPV 18 E7
      *  *    ** *     ***     * *  *** * **  *** **
PLKQHFQIVT   CCCGCDSNVRLVVQCTETDIREVQQLLLGTLNIVCPICAPKT HPV 6b E7
```

**Fig. 3**
Alignment of HPV-16, HPV-18 and HPV-6b E7 proteins
Amino acid sequences of 16/E7-5 and 18/E7-6 peptides are in bold italics. The program ALIGN developed by M. Stein and T. F. Smith, Los Alamos National Laboratory, and kindly distributed by Molecular Biological Computer Source was used.

engineered HPV-16 and HPV-18 proteins for identification of their B-epitopes. Our data indicate that the fifth peptide of HPV-16 E7 protein (aa 41–60) and the sixth peptide of HPV-18 E7 (aa 51–70) were broadly cross-reactive. When amino acid sequences of the two E7 proteins were compared, these two peptides matched as indicated in Fig. 3.

The aminoterminal peptide 18/E7-1 reacted with HPV-6/11 positive sera, but not with HPV-16 positive sera (Fig. 2), whereas HPV-16 E7-derived peptide 16/E7-1 did not show such cross reactivity (Krchňák *et al.*, 1990). Comparison of amino acid sequences did not indicate a much higher homology between types 6 and 18 than between types 16 and 18. However, the Chou-Fasman (1978) analysis of aminoterminal part of these three proteins revealed that the HPV-16 E7 protein has a much higher tendency for alpha helix formation when compared with the same region on HPV-6 and HPV-18 proteins. These two

proteins posses a higher overall beta sheet potential. The different conformational preference (alpha helix in type 16 versus beta sheet in types 6 and 18) may contribute to different recognition of type-specific and type-common antibodies.

The prevalence of antibody reactivity with the type-specific E7-derived peptides was low. Only 2 out of 65 INCA patients possessed HPV-18 E7 antibody and 3 of the 4 sera from HPV-18 DNA positive patients were free of this antibody. In this respect, our results seem to correspond to those reported by Bleul *et al.* (1991), who found only 3 out of 94 German INCA patients' sera reactive with a synthetic peptide comprising aa 32–59. On the other hand, a higher prevalence of E7 reactive antibodies was detected when bacterial fusion E7 protein was used as antigen (7 out of 46 sera from INCA patients were reactive; Kochel *et al.*, 1991).

In summary, we have identified seroreactive regions of HPV-18 E7 by testing overlapping peptides with pools of selected sera and used these peptides (18/E7-2, 18/E7-3, and 18/E7-5) to determine antibody presence against HPV-18 E7 protein in different groups of sera. We have demonstrated that the prevalence of antibodies reactive with these epitopes is very low. For monitoring HPV-18 infection, only peptide 18/E7-5 could possibly be used together with other, specifically reactive HPV-18-derived peptides which will probably be identified in the future.

## References

Barnes, W., Delgado, G., Kurman, R. J., Petrilli, E. S., Smith, D. M., Ahmed, S., Lorincz, A. T., Temple, G. F., Jenson, A. B., and Lancaster, W. D. (1988): Possible prognostic significance of human papillomavirus type in cervical cancer. *Gynecol. Oncol.* **29**, 267–273.

Bleul, C., Müller, M., Frank, R., Gausepohl, H., Koldovsky, U., Mgaya, H. N., Luande, J., Pawlita, M., TerMeulen, J., Viscidi, R., and Gissmann, L. (1991): Human papillomavirus type 18 E6 and E7 antibodies in human sera: Increased anti-E7 prevalence in cervical cancer patients. *J. clin. Microbiol.* **29**, 1579–1588.

Chou, P. Y., and Fasman, G. D. (1978): Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol.* **47**, 46–148.

Dillner, J. (1990): Mapping of linear epitopes of human papillomavirus type 16: The E1, E2, E4, E5, E6 and E7 open reading frames. *Int. J. Cancer* **46**, 703–711.

Jenison, S. A., Xiu-Ping, Y., Valentine, J. M., Koutsky, L. A., Kristiansen, A. E., Beckmann, A. M., and Galloway, D. A. (1990): Evidence of prevalent genital-type human papillomavirus infections in adults and children. *J. inf. Dis.* **192**, 60–69.

Köchel, H. G., Monazahian, M., Sievert, K. Höhne, M., Thomssen, C., Teichmann, A., Arendt, K., and Thomssen, R. (1991): Occurrence of antibodies to L1, L2, E4 and E7 gene products of human papillomavirus types 6b, 16 and 18 among cervical cancer patients and controls. *Int. J. Cancer* **48**, 682–688.

Krchňák, V., and Vágner, J. (1990): Color-monitored continuous – flow solid-phase multiple peptide synthesis. *Peptide Res.* **3**, 182–193.

Krchňák, V., Vágner, J., Šafář, P., and Lebl, M. (1988): Noninvasive continuous monitoring of solid-phase peptide synthesis by acid-base indicator. *Coll. Czech. chem. Commun.* **53**, 2542–2548.

Krchňák, V., Vágner, J., Suchánková, A., Krčmář, M., Ritterová, L., and Vonka, V. (1990): Synthetic peptides derived from E7 region of human papillomavirus type 16 used as antigens in ELISA. *J. gen. Virol.* **71**, 2719–2724.

Müller, M., Gausephol, M., DeMartynof, F. G., Frank, R., Brasseur, R., and Gissmann, L. (1990): Identification of seroreactive regions of the human papillomavirus type 16 proteins E4, E6, E7 and L1. *J. gen. Virol.* **71**, 2709–2717.

Selvey, L. A., Tindle, R. W., Geysen, M. H., Haller, C. J., Smith, J. A., and Fraser, I. H. (1990): Identification of B-epitopes in the human papillomavirus 18 E7 open reading frame protein. *J. Immunol.* **145**, 3105–3110.

Suchánková, A., Krchňák, V., Vágner, J., Krčmář, M., Ritterová, L., and Vonka, V. (1992): Epitope mapping of the human papillomavirus type 16 E4 protein by synthetic peptides. *J. gen. Virol.* **73**, 429–432.

Suchánková, A., Ritter, O., Hirsch, I., Krchňák, V., Vágner, J., Kaloš, Z., Hamšíková, E., Břicháček, B., and Vonka V. (1990): Presence of antibody reactive with synthetic peptide derived from L2 open reading frame of human papillomavirus types 6b and 11 in human sera. *Acta virol.* **34**, 433–442.

Tindle, R. W., Smith, J. A., Geysen, H. M., Selvey, L. A., and Frazer, I. H. (1990): Identification of B-epitopes in human papillomavirus (HPV) 16 E7 open reading frame (ORF) protein. *J. gen. Virol.* **71**, 2719–2724.

Walker, J., Bloss, J. D., Liao, S. Y., Berman, M., Bergen, S., and Wilcynski, S. P. (1989): Human papillomavirus genotype as a prognostic indicator in carcinoma of the uterine cervix. *Obstct. Gynecol.* **74**, 781–785.

Zur Hausen, H. (1989): Papillomaviruses as carcinomaviruses. *Adv. viral Oncol.* **8**, 1–26.