Arch Virol (2001) 146: 117–125

Archives of Virology

Egg yolk antibodies against the E7 oncogenic protein of human papillomavirus type 16

Brief Report

A. Di Lonardo[1,*], **M. Luisa Marcante**[1], **F. Poggiali**[1], **E. Hamsøíkovà**[2], and **A. Venuti**[1]

[1]Laboratory of Virology, Regina Elena Institute for Cancer Research, Rome, Italy
[2]Institute of Haematology and Blood Transfusion, Department of Experimental Virology, Prague, Czech Republic

Accepted August 2, 2000

Summary. The E7 oncoprotein is the major transforming protein of Human Papillomavirus type 16 (HPV16) and the most abundant in cervical neoplasia. In this study we report the production of polyclonal antibodies to HPV16 E7 in rabbits and hens. The produced antibodies recognised the denatured and native form of HPV16 E7 protein by Western Blot, and immunoprecipitation. Epitope mapping demonstrated that hen antibodies reacted with a greater number of antigen determinants than the rabbit antibodies. In immunocytochemistry only hen antibodies were able to localize the E7 protein in a HPV positive cell line and in the high-grade squamous intraepithelial lesions, suggesting their possible usefulness in the screening of clinical samples.

*

Cancer of the cervix is one of the most predominant neoplastic diseases in women, with a world-wide incidence second only to breast cancer. Epidemiological studies have established that infection with certain genital HPVs, most frequently HPV16, is by far the most significant risk factor for the development of high-grade squamous intraepithelial lesions (h-SIL), which can progress to invasive cervical carcinoma [6, 9, 22]. The E6 and E7 gene products are recognised as responsible for malignant cellular transformation and are consistently found in cervical cancer cells [19]. These oncoproteins can be targets for the development

*Present address: Laboratory of Virology – Istituto Superiore di Sanita, Viale Regina Elena 299, 00161 Rome, Italy.

of new diagnostic approaches to the disease, which could represent an alternative to HPV DNA hybridisation techniques. In particular, the HPV16 E7 gene product is the major viral transforming protein and the most abundant in cervical neoplasia [19].

Antibodies against HPV16 E7 proteins are specifically associated with cervical cancer and their serum level depends on tumour burden [2, 3, 5, 15]. Studies on HPV16 E7 antigen expression in neoplastic and dysplastic tissues and/or its possible correlation with serological response have been always hampered by the insufficient availability of large amount of specific antibodies to HPV16 E7 oncoprotein. A number of monoclonal antibodies against E7 protein or E7 derived peptides have been generated [17, 18, 20] and commercial preparations are also available, but they often suffer some disadvantages and to date they are not suitable for diagnostic purposes. Provision of polyclonal antibodies against the E7 oncoprotein of HPV16 could overcome all these limitations opening new perspectives in the diagnosis of HPV infection.

In this report, we describe the production of polyclonal antibodies to HPV16 E7 by using two different animal systems such as rabbits and chickens.

Three New Zealand White rabbits (Charles River, Como- Italy, 2,5 kg weight), aged 3 months, and three Padua Brown hens (from a local farmer), aged 5 months, were immunized with a purified Glutathione-S-Transferase-E7 HPV16 fusion protein (GST-E7) [3]. Primary immunisations were administrated in Freund's complete adjuvant, while boosters were in Freund's incomplete adjuvant. Antigen was injected subcutaneously in hen's breast and rabbit's back. Hens received 40 g of fusion protein at day 0 and two boosters of 20 μg each at day 12 and 20 as reported by Gassmann et al. [8]. Rabbits received 200 μg of antigen at first immunisation, and 100 μg, 60 μg, 60 μg of antigen at days 20, 35, and 49. Blood samples were obtained from ear vein of the rabbits 10 days after primary immunisation, and then at interval of 10–15 days. Eggs were collected daily and IgY were extracted according to the method described by Gassmann et al. [8].

The animal immune response was evaluated by Western Blot with immobilised bacterial GST-E7 fusion proteins as previously described [3]. Briefly, strips were incubated with rabbit sera and IgY preparations serially diluted from 1:10 to 1:100 000. Biotinylated anti-rabbit or anti-chicken IgG (Vector Laboratories, Inc., Burlingame, CA) at dilution 1:1000 were used as secondary antibodies, followed by addition of streptavidin-biotinylated horseradish complex for 30 min (Vector Laboratories, Inc., Burlingame, CA). Bound antibodies were visualised by a colorimetric or chemiluminescent assay (ECL) (Amersham Life Science, Buckinghamshire, England) and subjected to the densitometric scanning. The antibody specificity was confirmed by pre-absorption of hen and rabbit antibodies with GST-E7 fusion protein (Fig. 1).

IgY extracts and rabbit sera were tested by ELISA for their reactivity against eight peptides 20 amino acid (AA) each with 10 AA overlap and one peptide 28 AA long corresponding to the entire sequence of HPV16 E7 or the N terminal stretch, respectively [12]. IgY extracts were able to react with all of the nine peptides assayed even if with a different magnitude (Fig. 2a). In contrast, rabbit

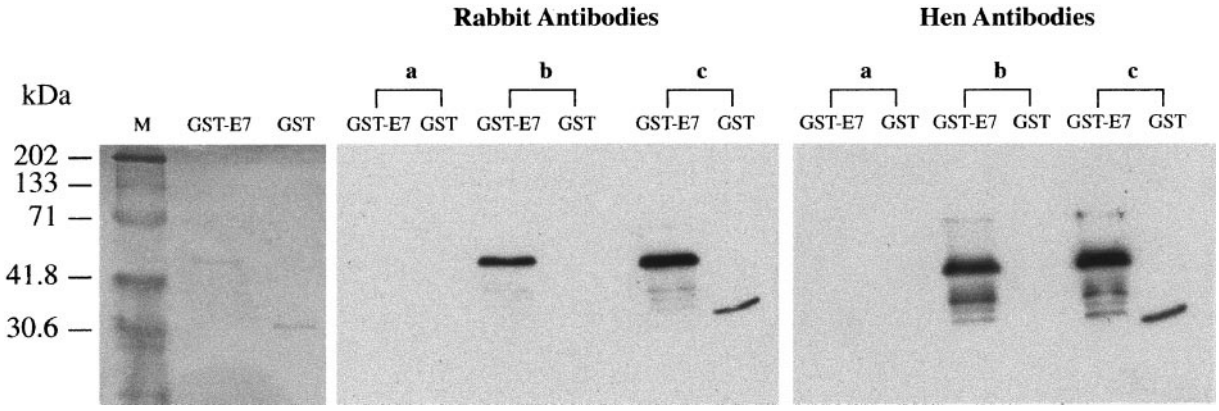

Fig. 1. Specificity of hen and rabbit antibodies by Western blot. GST E7 fusion and GST proteins were separated on 12% polyacrylammide gel and stained with coomassie blue (left panel). A counterpart of the gel was transferred onto nitro-cellulose membrane for Western Blot analysis with anti HPV16 -E7 rabbit and hen antibodies. Positive reaction was detected by ECL system. **a** Pre-absorption with GST-E7 protein; **b** pre-absorption with GST protein; **c** no-absorption

serum reactivity was limited to two peptides derived from the N-aminoterminal portion of the E7 protein, with E7/1 peptide presenting the highest score (Fig. 2b) Pre-immune preparations from both hens and rabbits did not react with any of these peptides (data not shown).

The ability of anti-HPV16 E7 hen and rabbit antibodies to recognise the HPV16 E7 protein expressed in vivo was investigated by Western blot and immunoprecipitation using protein extracts from CaSki and HaCaT cell lines. The former one is a human cervical cancer cell line expressing E7 protein from a 270–500 copies of HPV16 integrated DNA per cell [10], the latter one is a human keratinocyte cell line containing no HPV DNA sequences. In CaSki cells, anti-HPV16 E7 antibodies from both animals recognised in Western blot a band around 17–18 Kda, in accordance with the molecular weight reported by other authors [13, 21] (Fig. 3a). The hen antibodies showed a higher reactivity compared to rabbits as evidenced by the intensity of the specific band. Pre-incubation of anti-HPV16 E7 rabbit serum and anti-HPV16 E7 egg extracts with large amount of purified GST-E7 protein resulted in a loss of the band detection (data not shown).

Anti-HPV16 E7 hen and rabbit antibodies were also able to immunoprecipitate the native or the denatured form of HPV16 E7 protein expressed in CaSki cell line. As shown in Fig. 3b, both antibodies immunoprecipitated a protein of 17–18 Kda that was absent in HaCat extracts. The E7 protein precipitated by hen antibodies was also recognised by rabbit antibodies and vice-versa. The denatured form of HPV16 was detected, too (data not shown).

To extend the application range of the produced antibodies, immunocytochemistry was performed on CaSki, C33A cells and paraffin-embedded sections of HPV16 positive SILs biopsies. The two cell lines are derived from cervical carcinoma but C33A does not contain any HPV DNA sequences. The SILs biopsies

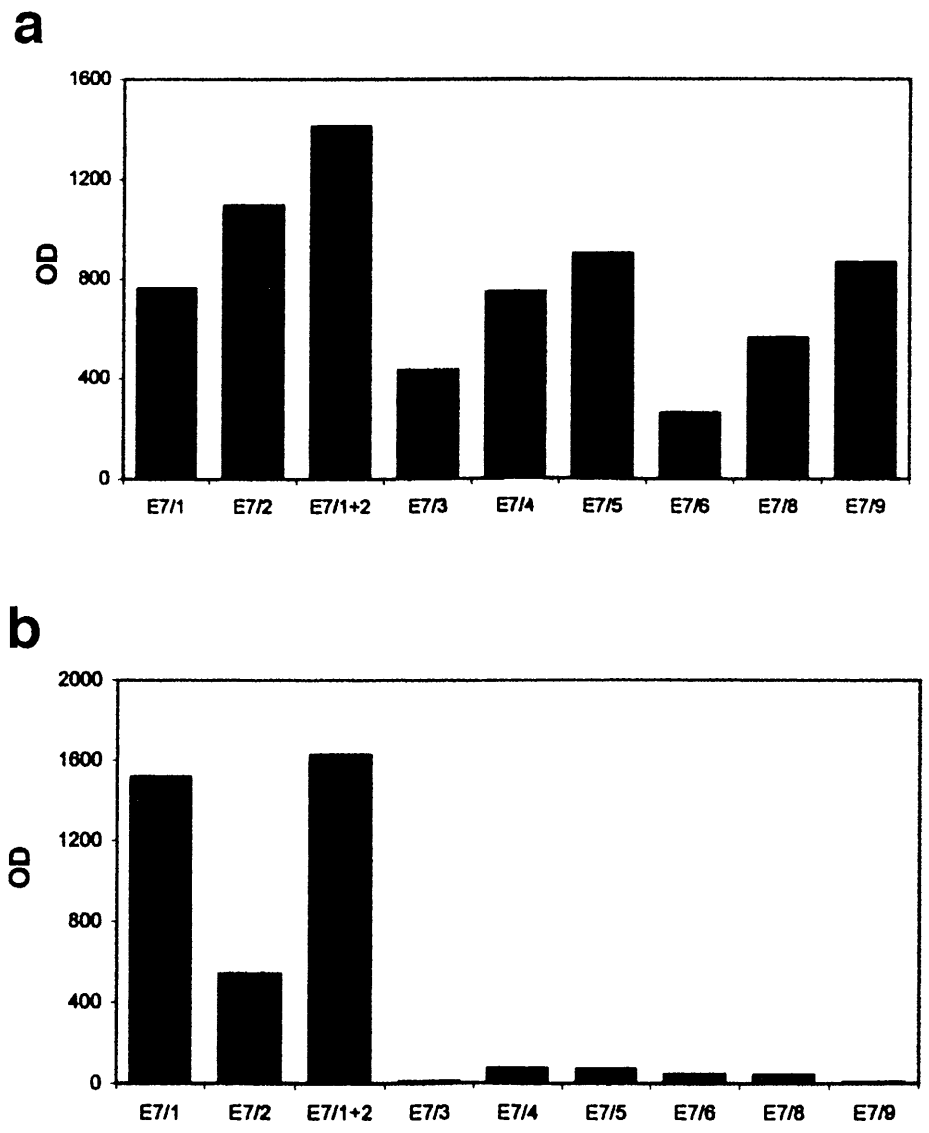

Fig. 2. Epitope mapping. Reactivity of hen (**a**) and rabbit (**b**) antibodies against a panel of overlapping peptides of HPV16 E7 region was tested in ELISA. Wells pre-coated with 2 ug of each peptide were incubated with 100 μl of 1:25 dilution of rabbit sera or IgY extracts in the buffer with 1% BSA. After extensive washings the plates were incubated with a 1:2000 dilution of peroxidase-conjugated anti rabbit IgG (SEVAC, Czech Republic) or anti chicken IgG (Pierce). Staining was performed with 0.04% o-phenylediamine and the extinction at 492 nm was measured using a microplate reader. Absorbance values were diminished by the absorbance values (< 10% of the maximum absorbance value) of negative rabbit serum or yolk extract

were chosen for the high levels of E7 mRNA detected [11]. The rabbit antibodies did not react with any cell line or histological section; in contrast, a strong specific immunoreactivity was observed in CaSki cells (Fig. 4b) assayed with anti-HPV16 E7 IgY, but not in C33A control cells (Fig. 4d). Positive staining was essentially

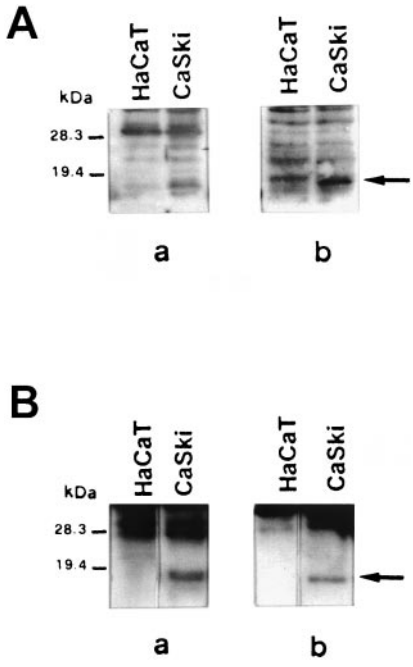

Fig. 3. Detection of E7 protein in cell lines. **A** Western blot analysis of E7 protein expressed in a HPV16 positive cell line. Eighty μg of protein extracts were fractionated by 12% SDS-PAGE, and transferred to PVDF membrane in 10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS) pH 11, 20% methanol by a trans-blot electrophoretic transfer cell apparatus (Bio-Rad). After blocking for 1 h in TBS-T buffer (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 05% Tween 20) containing 5% non-fat dry milk, filters were incubated overnight at 4 °C with a 1:50 dilution of anti-HPV16 E7 rabbit serum (*a*) or anti-HPV16 E7 IgY (*b*). After washings with TBS-T, filters were left to react with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Hercules CA) or horseradish peroxidase-conjugated rabbit anti-chicken IgG (Pierce, Rockford, IL USA). Positive reaction was detected by ECL system. The arrow indicates the position of E7 protein. **B** Immunoprecipitation of E7 protein expressed in a HPV16 positive cell line. One million of HaCaT and CaSki cells was lysed and pre-cleared cellular extracts were incubated with 5 μl of rabbit anti-HPV16 E7 serum (*a*) or anti-HPV16 E7 IgY (*b*) for 1 h at 4 °C. Rabbit immune complex was directly precipitated at 4 °C for 1 h by adding 40 μl of washed Protein A-Agarose, whereas hen immunocomplex was left to react with goat anti-chicken IgG (Pierce, Rockford IL) prior the addition of protein A-Agarose. Precipitated immunocomplexes were analysed as in Western Blot. The arrow indicates the position of the immunoprecipitated E7 protein

nuclear and at less extent cytoplasmic. In h-SIL histological sections, incubation with GST-pre absorbed anti-HPV16 E7 IgY produced a strong staining that was distributed throughout all the epithelium strata (Fig. 4g). In contrast, the incubation with GST-E7-pre-absorbed anti HPV16 E7 IgY completely abolished the staining on CaSki cells and on the histological sections (Fig. 4 c, f). Normal cervical epithelium tested with anti HPV16-E7 IgY was negative, too (4e). Likewise no reactivity was detectable on CaSki cell lines incubated with pre-immune IgY extracts (Fig. 4a).

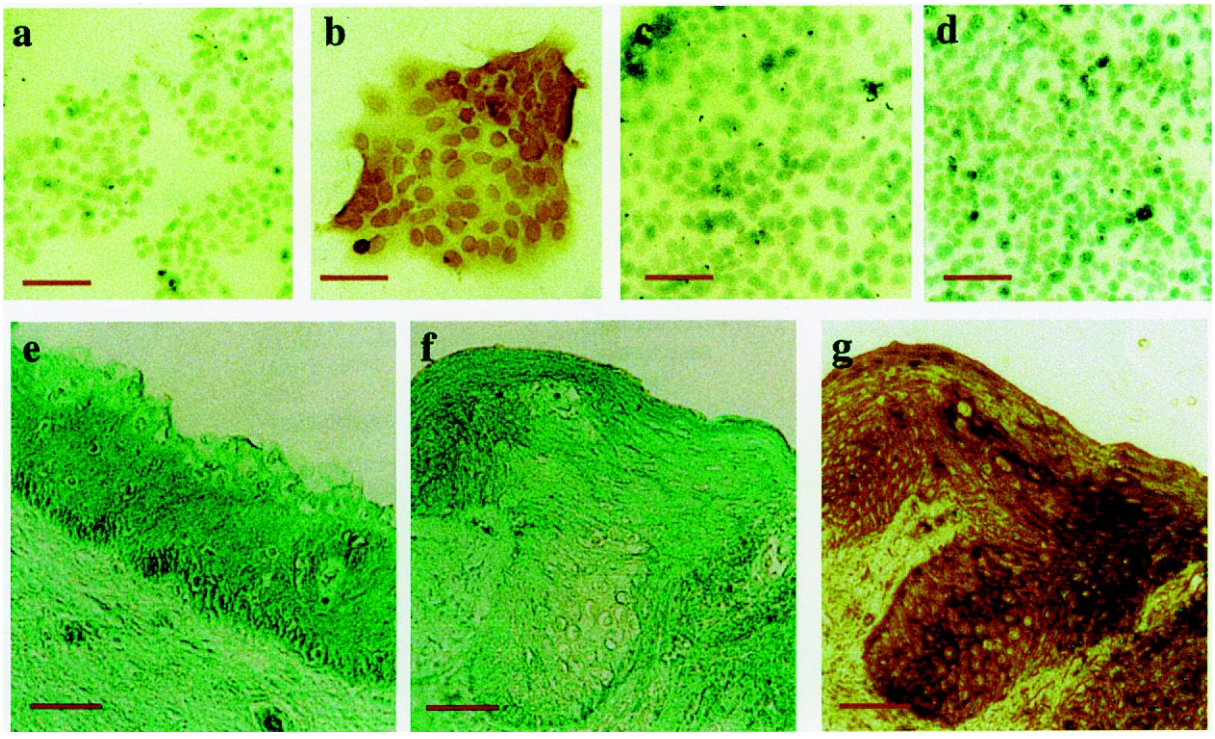

Fig. 4. Immunohistochemical detection of HPV16 E7 antigen in a HPV16 positive cell line and in a h-SIL biopsy. After the inhibition of the endogenous peroxidase by 0,3% hydrogen peroxidase/methanol solution, CaSki (**a, b, c**),C33A (**d**) cells, and a paraffin section of normal cervical epithelium (**e**) and HPV16 positive h-SIL (**f** and **g**) were incubated with 1:100 dilutions of pre- (**a**) or immune (**b, d, e**) egg yolk extracts, or with the IgY immune preparation after absorption to GST-E7 (c and f) or GST (**g**) protein. The immunoenzymatic reaction was visualised using DAB as chromogenic substrate and cellular counterstaining was performed with methyl green. Bars represent 100 μm

Our results indicate that hens and rabbits were both able to generate a specific anti-E7 response, but hens are more effective than rabbits. The major effectiveness in the antibody production by hens could be explained as result of a greater antigenicity of HPV16 E7 protein in this species, as compared to rabbit. Bollen et al. [1], employing human IgG as immunogen, reported that antibody production in chickens, when using the egg yolk as the antibody source, is a factor 5–10 times higher than that of the rabbit. Moreover, the simplicity of sampling, that is also not so labour intensive, avoids any distress for the animal, and finally costs of stabling are remarkably reduced [8].

The produced antibodies were able to recognise linear and/or conformational epitopes as demonstrated by using different immunoassays. Pre-absorption of hen extracts or rabbit antisera with GST-E7 proteins resulted in a loss of E7 band in Western blot confirming the specificity of the produced antibodies. The presence of a strong signal against GST-E7 even after the absorption with the GST protein clearly indicates that in both species a large amount of specific anti E7 IgG was produced (Fig. 1). These results were also strengthened by the presence of E7

band in Caski cells, containing high copy number of HPV16 DNA, and its absence in HaCat cells not expressing HPV protein (Fig. 3).

The epitope mapping, showing the presence of specific antibodies against HPV16-E7 protein, allowed to identify the different antigenic determinants. E7/1, E7/2 and E7/1+2 peptides, derived from N-terminal portion of HPV16 E7 protein were recognised by both hen and rabbit antibodies. These peptides were previously demonstrated to react specifically with sera from patients affected by invasive cervical cancer and positive for HPV16 DNA [12]. The E7/2 peptide, reported as the highest score peptide [12], was more reactive in hen than rabbit, suggesting a major ability of hen antibodies in detecting type specific HPV epitopes. The central protein (E7/5) and the C-terminal peptides of HPV 16 E7 were recognised only by hen antibodies. Epitope mapping results suggest that hen antibodies are able to recognise a greater number of antigenic determinants, some of them highly HPV specific.

All immunized hens produced polyclonal antibodies working in immunocytochemistry whereas the sera from all immunized rabbits did not react suggesting differences between the species. Therefore, in rabbit the production of antibodies against epitopes displayed on acetone or formalin fixed preparations, could not occur. This hypothesis is further strengthened by the differences found in the epitope mapping. The hen polyclonal antibodies detected the HPV16 E7 protein in CaSki cells and in HPV16 positive h-SIL histological sections with a strong staining in the nucleus and a slight one in the cytoplasm. No such staining was seen in C33A cells that are derived from a cervical carcinoma and do not contain any HPV DNA sequences, indicating the specificity of our antibodies also in immunocytochemistry.

The cytoplasmic localisation of HPV16 E7 protein is somewhat contradictory. Earlier studies by Sato et al. [16], and Meneguzzi et al. [14] showed a nuclear site of HPV16 E7, but they used transient expression systems such as Cos-1 cells and not cervical carcinoma cell lines. Subsequently, Fujikawa et al. [7], employing a mAb, detected a cytoplasmic staining in CaSki cells and in rat 3Y1 cells expressing HPV16 E7 gene. In contrast, hen anti-HPV16 E7 antibodies have the ability to recognise the nuclear as well as the cytoplasmic form of HPV16 E7 protein. On high-grade SIL biopsies we demonstrated the presence of HPV16 E7 proteins in all of epithelium layer. Other authors [4] reported a distribution of early HPV viral transcripts throughout all the epithelium in same genital lesions, even if with a strong positivity in basal cell layer, whereas Higgins et al. [11] detected a dramatic increase of the E7 signal in the superficial layers. Our results are in agreement with those of Stoler et al. [19] showing an E7 mRNA expression in all the epithelial strata.

Immunostaining with hen antibodies could be a new promising tool for the screening of HPV infection in clinical samples. Analyses on 60 low and high-SILs biopsies positive for HPV16 DNA by PCR, showed that anti-HPV16 E7 Ig Y were able to react in the majority of HPV16 positive cervical lesions, even if in a number of cases they were unable to detect the E7 protein (manuscript in preparation). This may indicate the absence or the extreme low level of E7

protein in some of the HPV positive lesions. Further histological testing along with RT-PCR for the presence of E7 mRNA, on a larger number of samples, will clarify this issue and will further validate these reagents.

In conclusion, the hen system seems more advantageous than rabbit in generating anti-HPV16 E7 antibodies against a broad spectrum of HPV16 epitopes that may allow to utilise them as a new potential tool for investigations of HPV16 E7 protein expression in clinical samples.

Acknowledgements

The authors wish to remember Mr. Luigi Dall'Oco that recently passed away, whose graphic and computing assistance was precious for this work. The authors are grateful to Mr. Antonio Muller for technical assistance, to Mr. Giuseppe Bertini for animal handling service. The authors are also in debt with Dr. Marco Giorgio Paggi for critical reading of the manuscript and with Prof. Vladimìr Vonka for his interest in the work. This work was partially supported by AIRC and CNR-Biotechnology Project, and by grant N. 4070-3, Granting Agency of Czech Ministry of Health.

References

1. Bollen LS, Crowley A, Stodulski G, Hau J (1996) Antibody production in rabbits and chickens immunized with human IgG: A comparison of titre and avidity development in rabbit serum, chicken serum and egg yolk using three different adjuvants. J Immunol Methods 191: 113–120
2. Di Lonardo A, Campo MS, Venuti A, Marcante ML (1994) Antibody response to E6, E7, and L1 proteins of Human Papillomavirus 16 in an Italian population. J Med Virol 43: 357–361
3. Di Lonardo A, Marcante ML, Poggiali F, Venuti A (1998) HPV16 E7 antibody levels in cervical cancer patients: before, and after treatment. J Med Virol 54: 192–195
4. Dürst M, Glitz D, Schneider A, zur Hausen H (1992) Human Papillomavirus type 16 (HPV16) gene expression and DNA replication in cervical neoplasia: analysis by in situ hybridisation. Virology 189: 132–140
5. Fisher SG, Benitez-Bribiesca L, Nindl I, Stockfleth E, Muller M, Wolf H, Perz-Garcia F, Guzman-Gaona J, Gutierrez-Delgado F, Irvin W, Gissmann L (1996) The association of human papillomavirus type 16 E6 and E7 antibodies with stage of cervical cancer. Gynecol Oncol 61: 73–78
6. Franco EL (1995) Cancer causes revisited: human papillomavirus and cervical neoplasia. J Natl Cancer Inst 87: 779–780
7. Fujikawa K, Furuse M, Uwabe K, Maki H, Yoshie O (1994) Nuclear localization and transforming activity of human papillomavirus type 16 E7-β-galactosidase fusion protein: characterization of the nuclear localization sequence. Virology 204: 789–793
8. Gassmann M, Thommes P, Weiser T, Hubscher U (1990) Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. FASEB J 4: 2528–2532
9. Gissmann L (1992) Human papillomaviruses and genital cancer. Semin Cancer Biol 3: 253–261
10. Herrington CS, Burns J, Graham AK, Evans M, Mc Gee, JO (1989) Interphase cytogenetics using biotin and digoxigenin labelled probes I: relative sensitivity of both reporter molecules for detection of HPV16 in CaSki cells. J Clin Pathol 42: 592–600
11. Higgins GD, Uzelin DM, Phillips GE, McEvoy P, Marin R, Burrell CJ (1992) Transcription patterns of human papillomavirus type 16 in genital intraepithelial neoplasia:

evidence for promoter usage within the E7 open reading frame during epithelial differentiation. J Gen Virol 73: 2047–2057
12. Krchnak V, Vagner J, Suchankova A, Krcmar M, Ritterova L, 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
13. Imai Y, Matsushima Y, Sugimura T, Terada M (1991) Purification and characterization of human papillomavirus type 16 E7 protein with preferential binding capacity to the underphosphorylated form of retinoblastoma gene product. J Virol 65: 4966–4972
14. Meneguzzi G, Cerni C, Kieny MP, Lathe R (1991) Immunization against human papillomavirus type 16 tumor cells with recombinant vaccinia viruses expressing E6 and E7. Virology 181: 62–69
15. Muller M, Viscidi RP, Sun Y, Guerrero E, Hill PM, Shah F, Bosch FX, Munoz N, Gissmann L, Shah KV (1992) Antibodies to HPV16 E6 and E7 proteins as markers for HPV-16 associated invasive cervical cancer. Virology 187: 508–514
16. Sato H, Watanabe S, Furuno A, Yoshiike K (1989) Human papillomavirus type 16-E7 protein expressed in Escherichia coli and monkey cos-1 cells: immunofluorescence detection of the nuclear E7 protein. Virology: 170: 311–314
17. Selvey LA, Dunn LA, Murray B, Tindle RW, Frazer IH (1992) An ELISA capture assay for the E7 transforming proteins of HPV16 and HPV18. J Virol Methods 37: 119–127
18. Stacey SN, Eklund C, Jordan D, Smith NK, Stern PL, Dillner J, Arrand JR (1994) Scanning the structure and antigenicity of HPV-16 E6 and E7 oncoproteins using antipeptide antibodies. Oncogene 9: 635–645
19. Stoler MH, Rhodes CR, Whitbeck A, Wolinsky SM, Chow LT, Broker TR (1992). Human papillomavirus type 16/18 gene expression in cervical neoplasias. Hum Pathol 23: 117–128
20. Tindle RW, Zhou WD, Saul A, Frazer IH (1990) The molecular specificity of linear B-epitopes in the E7 open reading frame protein of human papillomavirus 16 defined by monoclonal antibodies. Peptide Res 3: 162–166
21. Zerfass K, Levy LM, Cremonesi C, Ciccolini F, Jansen-Durr P, Crawford L, Ralston R, Tommasino M (1995) Cell cycle-dependent disruption of E2F-p107 complexes by human papillomavirus type 16 E7. J Gen Virol 76: 1815–1820
22. zur Hausen H (1996) Papillomavirus infections – a major cause of human cancers. Biochim Biophys Acta 1288: F55–F78

Authors' address: Dr. A. Venuti, Laboratory of Virology, Regina Elena Institute for Cancer Research, Via delle Messi d'Oro, 156-00158 Rome, Italy.

Received 28 February, 2000