THE HUMORAL IMMUNE RESPONSE AGAINST THE TRANSFORMING PROTEINS E6 AND E7 OF HUMAN PAPILLOMAVIRUS TYPE 16 IN PATIENTS WITH SQUAMOUS CELL CARCINOMA OF THE UTERINE CERVIX

Clinical assessment of the diagnostic and prognostic value

DE HUMORALE IMMUUNRESPONS TEGEN DE TRANSFORMERENDE EIWITTEN E6 EN E7 VAN HUMAAN PAPILLOMAVIRUS TYPE 16 IN PATIËNTEN MET BAARMOEDERHALSKANKER

Klinische evaluatie van de diagnostische en prognostische waarde

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CARCINOMA OF THE UTERINE CERVIX AND HUMAN PAPILLOMAVIRUS

1.1. Carcinoma of the uterine cervix

Incidence

Cervical cancer is one of the most common cancers worldwide and is responsible for 12% of all cancers in women, which equals to 437,000 new cases in 1985[156]. In developing countries cervical carcinoma is the most frequent malignancyin females. In the developed world it accounts for 6% of all cancers in females[156], with approximately 750 new cases and 275 deaths yearly in the Netherlands[228].

Histology of the normal cervix

The cervix comprises two distinct parts; the ectocervix, which is covered by stratified non-keratinizing squamous epithelium, and the endocervix, covered by mucin-secreting columnar epithelium. The transition area of these two epithelia is situated at the level of the external os and is a zone of metaplastic squamous epithelium, which is referred to as the transformation zone. The border between the metaplastic epithelium and the glandular epithelium is termed the neosquamocolumnar junction. With increasing age, the glandular epithelium is pushed back into the endocervical canal by the squamous ectocervical epithelium. Almost all cervical squamous neoplasia begin at the transformation zone.

Precursor lesions of cervical carcinoma

The notion that cervical cancer is preceded by precursor lesions stems from the first half of this century. A temporal relationship between carcinoma *in situ* and invasive cancer was reported[158,191]. Long-term follow-up studies of untreated patients with carcinoma *in situ* showed that a significant portion of these patients developed invasive squamous cell carcinoma[114,115]. In 1956 Reagan *et al.* coined the term dysplasia to include the whole spectrum from mild epithelial changes to carcinoma *in situ*[167]. In the late 1960s Richart introduced the concept that all precursor lesions leading to carcinoma *in situ* represent a single continuous disease process, which was subsequently termed cervical intraepithelial neoplasia or CIN[170,171]. The CIN terminology subdivides precursor lesions into three classes, ranging from mild dysplasia (CIN 1, up to 1/3 of the epithelium involved), via moderate dysplasia (CIN 2, up to 2/3 involved) to severe dysplasia and carcinoma *in situ*

(CIN 3, more than 2/3 thickness involved).

In 1988 the Bethesda System suggested the terms low grade and high grade squamous intraepithelial lesions (L-SIL and H-SIL), where L-SIL is CIN 1 and flat condylomata, and H-SIL combines CIN 2 and CIN 3[128,151].

Invasive cervical carcinoma

There are three general categories of epithelial tumours of the cervix, namely squamous cell carcinoma, adenocarcinoma and other epithelial carcinomas. The first group accounts for 60-80% of all invasive carcinomas of the cervix. The carcinomas are staged according to the system of the International Federation of Gynaecologists and Obstetricians (FIGO). This system divides invasive carcinoma into four stages (Table 1). Stage 0 is carcinoma *in situ*. Stage I includes all tumours confined to the cervix, and is subdivided into two categories; microinvasive and more deeply invasive. Stage II tumours extend beyond the cervix but not to the pelvic sidewall and do not involve the lower third of the vagina. Stage III tumours include those that extend to the pelvic sidewall, cause hydronephrosis or a non-functioning kidney or involve the lower third of the vagina. Stage IV tumours extend beyond the true pelvis or involve the mucosa of the bladder or rectum[232].

Table 1. Staging of cervical cancer.

Stage		Definition
0		Carcinoma *in situ*, not invasive
I		The carcinoma is strictly confined to the cervix
	Ia	Invasive cancer identified only microscopically, invasion is limited to measured stromal invasion with a maximum depth of 5mm and no wider than 7mm
	Ib	Lesions of greater dimension than Ia
II		The carcinoma extends beyond the cervix, but has not extended on to the pelvic wall; the carcinoma involves the vagina, but not as far as the lower third
	IIa	No obvious parametrial involvement
	IIb	Obvious parametrial involvement
III		The carcinoma has extended on to the pelvic wall; on rectal examination there is no cancer-free space between the tumour and the pelvic wall; the tumour involves the lower third of the vagina; all cases with hydronephrosis or non-functioning kidney are included
	IIIa	No extension to the pelvic wall, but involvement of the lower third of the vagina
	IIIb	Extension to the pelvic wall or hydronephrosis or non-functioning kidney
IV		The carcinoma has extended beyond the true pelvis or has clinically involved the mucosa of the bladder or rectum
	IVa	Spread of the growth to adjacent organs
	IVb	Spread to distant organs

Mass screening for cervical cancer and its precursor lesions

Mass screening for precursor lesions of cancer of the cervix is based on the premise that there is a natural progression from a normal cervical epithelium through intraepithelial neoplasia to invasive cancer of the cervix. If individuals could be identified and treated early in this process, progression to invasive disease could be prevented[149]. Cytological examination of exfoliated cells stained according to the Papanicolaou method has first been introduced for mass screening in 1949, in British Columbia[66,67]. The cervical smear appears to be prone to sampling and reading errors, with considerable false-negative rates as a consequence. Inter-observer variation have been reported[24,220]. Screening has proven successful to reduce both the FIGO stage upon finding the lesion, and the number of deaths resulting from cervical cancer[11,13,19,62,99,155]. The risk of dying of cervical cancer is ten times higher in non-participants of screening programs as compared to participants[100].

Tumour markers for cervical carcinoma

For patients with frank invasive cervical carcinoma tumour markers which correlate with tumour burden and give insight in the clinical course of disease (prognosis) are of particular value. The ideal tumour marker would be expressed by all cells within a tumour and be released in large quantities into a body compartment which can easily be sampled. In the case of cervical cancer a number of tumour markers have been proposed, such as carcinoembryonic antigen (CEA), keratins, squamous cell carcinoma antigen (SCC-ag) and cancer antigen 125 (CA 125).

For CEA, an oncofetal antigen, good correlation between tumour burden and initial plasma values has been reported. Serial determinations during follow-up enable early detection of known recurrence in squamous cell carcinoma[112,209,224].

Keratins represent a group of intermediate filament proteins in epithelial cells, which provide internal stability to the cells. Some keratins, namely 8, 14, 17, 18 and 19, are expressed by the majority of cervical carcinomas[194]. Of some of these cytokeratins fragments are released and can appear in serum. These fragments can be detected in assays for fragments of cytokeratins 8, 18 and 19 (tissue polypeptide antigen or TPA[140]) or cytokeratin 19 alone (CyFra 21-1[17]). A strong relation between Cyfra 21-1 level and tumour size was found, as well as a relation with disease-free interval and survival[18].

SCC-ag is a purified fraction of TA-4, which is a tumour antigen extracted from a human cervical squamous cell carcinoma[104,107]. Detailed studies have made it clear that the serum determination of SCC-ag is a valuable diagnostic tool in monitoring therapy, and

follow-up of patients with squamous cell carcinoma of the cervix[60,105,106], but not in patients with adenocarcinoma[59,142].

Finally, CA 125 was first detected after the preparation of monoclonal antibodies against a serous cystadenocarcinoma of the ovary[8]. It has been shown that CA 125 is also a good marker for patients with adenocarcinoma of the cervix[57-59].

Prognostic factors

Generally, therapy of cervical cancer is based on FIGO classification of the tumour. The 5-year survival for treated stage I patients is 90-95%, for stage II 50-70%, 30% for stage III and less than 20% for patients with stage IV disease[232]. In large studies, stage Ib and IIa patients are often evaluated together, and a combined 5-year survival rate of 80% has been reported. These patients can be treated with surgical or radiation therapy, with comparable results[219].

It is comparatively easy to obtain data on tumour characteristics after surgery, but not after radiation therapy. As surgery is normally limited to patients with lower FIGO stages, data on tumour characteristics in patients with higher FIGO stages are scarce. Some 20% of the patients in FIGO stages Ib/IIa die of cervical cancer, due to failure of local therapy or untreatable distant metastases. The evaluation of tumour characteristics may help in identifying those patients in need of further therapy.

Possibly the most important prognostic factor is involvement of lymph nodes. Involvement of pelvic lymph nodes has been reported in an average of 17% of Stage Ib/IIa patients with lower incidences for common iliac and paraaortic lymph nodes (3-5%). The presence of positive lymph nodes reduces the 5-year survival rate to 50%[69,73,219].

The differentiation grade of cervical cancer cells has also been shown to possess prognostic value. Patients with well-differentiated lesions show a significantly better 5-year survival rate compared to patients with poorly differentiated tumours[14,46,94], as well as fewer lymph node metastases and recurrences[32,69,195].

Similarly, it has been shown that increasing tumour volume is associated with increased incidence of lymph node metastases and recurrences, as well as a decreased 2-year survival[31,69].

Vascular invasion, whether of lymphatic or blood vessels, is also positively correlated with lymph node involvement and recurrence of disease. The 5-year survival rate is significantly worse for patients with vascular invasion[22].

Finally, depth of cervical invasion is also correlated to the frequency of lymph node

metastasis, recurrences, and the survival rate[22,31,69].

As these factors are interrelated, it is difficult to pinpoint the most important factor. Nevertheless, these factors appear to allow prediction of survival, and suggest that the FIGO classification alone is insufficient for subdividing groups of patients for therapy. Patient selection according to tumour characteristics described above may allow therapy modifications and improve survival[219].

Etiology

A large number of risk factors have been described for both cervical cancer and its precursor lesions. The most important risk factors are related to sexual activity, being life-time number of sexual partners[160,193], age at first sexual intercourse[118,159], and characteristics of the male sexual partner[23,111]. This suggestion that cervical cancer is a sexually transmitted disease spurred the search for a pathogenic agent. Among the list of organisms proposed as etiological agents are *Chlamydia trachomatis*, *Treponema pallidum*, *Trichomonas vaginalis*, *Neisseria gonorrhoea*, cytomegalovirus and herpes simplex virus (HSV). However, none of these pathogens have shown a strong correlation with cervical cancer, with the possible exception of HSV-2[88,141,193].

It was not until 1976 that the human papillomavirus (HPV) was implicated as the primary etiological agent in the pathogenesis of cervical cancer[241].

1.2. Human papillomavirus

Introduction

The papillomaviruses together with the polyomaviruses form the papovavirus family. This name was derived from the members of this family; the *pa*pillomaviruses, the *po*lyomaviruses and simian *va*cuolating virus. All viruses in this family are small in size, have a non-enveloped virion and a double stranded DNA genome. However, the differences in genomic organisation and biology between papillomaviruses and polyomaviruses has led to a division of the two viruses in separate subfamilies of the papovaviruses.

Papillomavirus particles are 52-55 nm in size. The particles contain a single molecule of double stranded circular DNA of approximately 8000 basepairs. The capsid of the particle is composed of 72 capsomeres, which form an icosahedral structure.

The papillomaviruses are highly host- and tissue-specific and induce proliferative squamous epithelial and fibroepithelial lesions in their natural hosts. The life cycle is

closely linked to keratinocyte differentiation. In basal epithelial cells the viral genome is maintained as a multicopy episome. It has long been known that certain papillomaviruses can cause cancers, dating back to the discovery in the early 1930s that the cotton tail rabbit papillomavirus is associated with tumour formation in its natural host[177,192].

HPV taxonomy

Since the molecular cloning of HPV type 1 in 1980[83] more than 70 different HPV types have been identified[50]. New types are defined as having less than 50% cross-hybridization to known HPV types by reassociation kinetics. With the widespread use of the polymerase chain reaction (PCR) this definition has been modified; the nucleotide sequence of the E6, E7 and L1 open reading frames (ORF) should demonstrate less than 90% sequence identity with known HPV types to qualify as a new type[51].

All human papillomaviruses are strictly epitheliotropic, with a division in cutaneous and mucosal types. Most cutaneous types are associated with benign warts and wart-like lesions, some types can also be found in skin cancer in patients with epidermodysplasia verruciformis (EV). The mucosal types infect the anogenital and aerodigestive tract. They are subdivided in low risk types, e.g. 6 and 11, which are associated with benign lesions such as condyloma acuminata, and high risk types, e.g. 16 and 18, which are associated with CIN and cervical cancer. Some reports also distinguish intermediate risk types, e.g. 31 and 33.

Phylogenetic analysis of all presently known HPV based on homology in nucleotide and/or amino acid sequences confirms the forementioned division in cutaneous and mucosal types, and also the subdivision in low risk and high risk mucosal types. Furthermore, cutaneous types causing EV form a separate branch[15,26,226]. However, all groups contain some anomalies.

Genomic organisation

The viral genome of HPV-16 (figure 1.1) is organized into three regions; an early region of 4.5 kbp, a late region of 2.5 kbp and a non-coding region (NCR) of 1 kbp. The latter region is also called upstream regulatory region (URR) or long control region (LCR), and contains the origin of replication and control elements for transcription and replication. The late region codes for the two late proteins, L1 and L2, which form the capsid. L1, which is the major capsid protein, is about 55 kDa and accounts for 80% of the total viral protein. This protein is highly conserved among HPV. L2, the minor capsid protein, is about 70

kDa and is much more variable among viral types. It has been shown in *in vitro* studies that L1 alone can form virus like particles (VLP)[109,119], but the presence of L2 seems to enhance the stability of the VLP[80,110,239]. Furthermore, L2 protein binds HPV DNA and may play a role in capsid assembly by introducing HPV DNA into the virus particle[240].

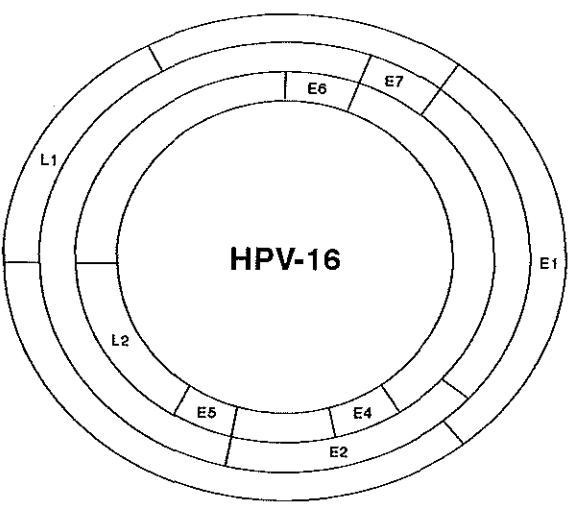

Figure 1.1. Depiction of the HPV-16 genome. The approximately 8 kb double-stranded HPV genome encodes 6 early ORF proteins (E1, E2, E4, E5, E6 and E7) and 2 late ORF proteins (L1 and L2) on a single strand of circular DNA (adapted from 216).

The early region contains the ORF for E1, E2, E4, E5, E6 and E7. E3 and E8 are only found in animal PV. The E1 protein has a function in viral DNA replication[77,208]. Most work has been done on bovine papillomaviruses (BPV). In BPV 1, E1, together with E2, downregulates the E5, E6 and E7 oncogenes[225]. The E2 ORF encodes at least two proteins with different regulatory functions, a full-length protein and a truncated form. In all papillomaviruses E2 binding sites (E2BS, also called E2-responsive element or E2RE) with the sequence $ACCN_6GGT$ are present[74,81,90,134,201]. These E2BS will bind both the full-length E2 and the truncated form. Full-length E2 protein is a potent transcriptional activator of viral gene expression through binding of the two E2 binding sites located close to the TATA box of the P97 promotor of HPV 16. The C-terminal half of the E2 protein is a

weak repressor of viral gene expression[21,210], most likely through direct competition for E2BS.

The E4 protein is expressed as a E1^E4 fusion protein encoded by a short segment of the E1 ORF linked to the E4 ORF, yielding a protein of 10 kDa. Although the E4 gene is situated in the early region, it appears to be a late protein which colocalizes with the L1 protein. Expression of E4 may facilitate release of new virions by collapsing the cytokeratin skeleton[56]. For binding of E4 to cytokeratin a conserved motif, LLXLL (X being any amino acid), seems to be essential[172].

The E5 ORF encodes a 10 kDa protein which localizes to the Golgi apparatus and the plasma membrane and can form complexes with a number of transmembrane proteins[37]. The BPV E5 analogue is a transforming protein, which is thought to function through binding and activation of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors[164].

Transforming proteins E6 and E7 are both small proteins that show some similarity to each other. It has been proposed that these proteins have arisen following amplification and divergence of a DNA sequence coding for a 33 amino acid peptide[34]. As opposed to the low-risk types, which can all only produce full-length E6 transcripts, the E6 ORF of high risk HPV types (HPV 16, HPV 18) can code for full-length E6 and for truncated E6* transcripts, owing to internal splicing of some of the transcripts[185,199]. In contrast to the full-length E6 and E7, the E6* protein is generally not detected in cervical carcinoma cell lines[188]. It was shown that full-length E6, but not E6*, can induce anchorage-independent growth and trans-activate the adenovirus E2 promoter[187]. Furthermore, the generation of a mutation in the splice site severely reduced E7 protein levels and increased E6 protein levels. This suggests that the splicing event regulates the level of translation of the E7 protein and that E6* is merely a by-product of this reaction[187]. The E7 ORF encodes a 21 kDa phosphoprotein which is constitutively expressed in cervical carcinomas and cancer-derived cell lines[4,185,199]. Like E6, E7 shows high affinity zinc binding through Cys-X-X-Cys repeats[7,136,157,166].

In low-grade squamous lesions, all viral ORF are expressed, the most abundant transcript spanning the E4 and E5 ORF[206]. L region transcription is restricted to terminally differentiated keratinocytes. As the grade of the lesion increases, cellular differentiation and viral transcription decrease, with the exception of the E6-E7 region[206]. HPV 16 associated cervical carcinoma cells will still express E2-E4-E5 at a low level, whereas HPV 18 infected cells seem to loose the ability to express the distal portions of the E region,

indicating complete integration of HPV 18 sequences into the cellular genome[206].

Detection of HPV DNA

Epidemiological studies on HPV prevalence have been performed using nucleic acid hybridization techniques. Southern blot hybridization has been proclaimed as the "Gold Standard" because of its sensitivity and specificity. However, major drawbacks of Southern blot hybridisation are the amount of material required, and the fact that the technique is time-consuming, which renders it unsuitable for mass screening purposes. Other techniques, such as filter *in situ* hybridization, dot blot hybridization or liquid hybridization, are less labour-intensive, but lack the sensitivity and specificity of Southern blot analysis. Early epidemiological studies reported conflicting results because of the variation in techniques, and the accompanying problems with sensitivity and specificity.

The development of the PCR technique[180] has provided an assay which is much more sensitive, with the theoretical ability to detect a single copy of HPV DNA per sample. Furthermore, DNA isolation is not necessary as PCR can be performed on crude cell suspensions[222] and also on archival material such as paraffin-embedded tissue[191] and archival cervical smears[168,198].

For the detection of any mucosatropic HPV type in a sample the use of a single primer pair would be ideal. Several different consensus primer pairs have been developed, either as degenerate primers[78,130,211,236] (multiple primers with slightly differing DNA sequences, used with high annealing temperatures), or as mismatch acceptance primers[200] (a single DNA sequence, used with a low annealing temperature). Using these primer pairs in PCR, several interesting results have been obtained. First of all, in a normal and healthy population there is a relation between presence of HPV and age, with a reduction in HPV prevalence after the age of 35[49,137,139]. Furthermore, there is a linear association between HPV prevalence and increasing Pap class[233], or CIN lesion[10,38] , culminating in an HPV prevalence of well over 90% in invasive cervical carcinoma[102,168,221,236].

Role of HPV in cervical carcinogenesis

As HPV cannot be grown in cell culture, direct evidence for the role of HPV in carcinogenesis is limited. Transformation of the murine fibroblast cell line NIH 3T3 with HPV 16 DNA renders these transformed cells tumorigenic in nude mice[234]. In a cotransfection assay of baby mouse kidney cells, DNA from HPV types 16, 18, 31 and 33 cooperates with ras or fos oncogenes to produce cell lines that are tumorigenic in

immunocompetent mice[41,132]. Both human primary fibroblasts and keratinocytes can be transformed with HPV 16 DNA[165]; transformed fibroblasts show an extended life-span, whereas keratinocytes, the HPV target cell, show an indefinite life-span. Both transformed cell-types express HPV-16 specific mRNA[165]. Infection with the HPV 16 genome of a cell culture system for growing keratinocytes results in the production of histological abnormalities identical to those normally seen in intraepithelial neoplasia *in vivo*[135]. E6-E7 oncoprotein transcripts can be identified on Northern blot, indicating that these cells express the HPV 16 transcripts similar to those seen in cervical carcinomas *in vivo*[135].

Mutations in either E6 or E7 genes abrogate transformation of primary human foreskin keratinocytes completely, whereas mutations in E1, E2 and E4 do not seem to affect transformation suggesting translational integrity of full-length E6 and E7 is necessary and sufficient for transformation of primary human keratinocytes[146,187].

There is little doubt that infection with an oncogenic or high risk HPV type (16, 18, 31, 33, etc.) is a major risk factor for cervical neoplasia[116,184]. However, epidemiological evidence has clearly shown HPV infection alone is not sufficient for progression to cancer. It would seem that the HPV-infected cell has to undergo additional genetic changes. One of the changes which have been proposed as a cofactor in carcinogenesis is association of HPV-encoded proteins with tumour suppressor genes. The E6 protein binds the p53 tumour suppressor protein[121,230], which in its functional form is associated with the ability of cells to arrest in G1 phase after DNA damage[126]. Binding of p53 by E6 is promoted by a cellular protein, called E6-associated protein (E6-AP, 100 kDa[92,93]) and renders p53 vulnerable to the ubiquitin-mediated proteolytic system[6,217]. Binding efficiency of E6 to p53 seems to be related to oncogenic potential, HPV 16 displaying highest efficiency, followed by HPV 31, HPV 18 and HPV 11[122]. C-terminal E6 mutants do not bind p53 and therefore fail to initiate degradation, whereas N-terminal E6 mutants can bind p53, but are unable to direct degradation[42,131]. Interaction of E6 with a number of other cellular proteins, including a protein kinase, has been reported[108]. The E7 protein binds to the retinoblastoma gene product p105-Rb[61]. This product has been shown to possess anti-oncoprotein activity. Binding of p105-Rb has also been shown for SV40 large T antigen[47] and adenovirus E1A[231]. This suggests that these three DNA viruses utilize similar mechanisms for transformation[61,162,163]. The efficiency with which E7 oncoproteins bind the p105-Rb correlates with the classification as high or low risk type[82,147]. Initially, the biochemical and biological differences could be assigned to the amino terminal sequences[148], but the crucial difference between HPV 16 E7 and HPV 6 E7 seems to lie in a single amino acid variation,

which was shown to be responsible for the divergence in p105-Rb binding and hence the transforming potential[82,181]. As for E6, E7 has been shown to associate with other cellular proteins, including cdk2 and cyclin A[217].

Another possible co-factor in carcinogenesis is integration of the viral genome into the cellular genome[4]. DNA analysis of cervical carcinoma derived cell lines has shown that a large number contains HPV DNA in integrated forms, both in long established cell lines[4,186,235] as well as in four cell lines recently derived from cervical carcinomas[202], suggesting that integration of HPV 16 DNA in long established cell lines is not due to selection *in vitro*. The integration event appears to be random with respect to the host genome, but specific with respect to the viral genome, almost always taking place near the E1-E2 boundary[4,197,206]. Disruption of the E2 ORF would interfere with its transcriptional regulatory function, leading to an increased expression of E6 and E7. Although integration is much more frequent in cervical cancer than in precursor lesions[43,44,123], around 30% of HPV 16 associated primary cervical cancers will contain extrachromosomal DNA only[27,43,44,68,133], implying that integration is not required for malignant progression in HPV 16 carcinomas. As is the case with HPV 5 in EV, an episomal form of HPV 16 might be sufficient to introduce a fully malignant phenotype. HPV 18 associated cancers all show integration into the cellular genome[43,206].

Finally, the immune response seems to play a role in progression of precursor lesions to cervical cancer, as immunosuppressed patients are at higher risk to develop anogenital neoplasias[63,138,152,154,161]. The immune system and its (dys)function in HPV infection is described hereafter.

1.3. Immunity to human papillomavirus

1.3.1. Immunity to viruses

Viruses lack the apparatus necessary for reproduction and, therefore, have to infect cells and take over the cell's replicative machinery. The host's immune response functions to prevent the spread of the virus in the body. This response is based on two pathways; the innate immune response and the acquired immune response. The innate arm consists of the natural killer cells (NK cells) and macrophages. Furthermore, virus-infected cells can secrete interferon (IFN). On binding to receptors on uninfected neighbouring cells, interferon will render the cell resistant to infection. The acquired immune response can be divided in the cell-mediated and the humoral immune response.

Cellular immune reponse to viruses

The cellular immune response is mediated by a number of cells, notably T helper, T suppressor and cytotoxic T-cells. In the case of viral infections, the cell-mediated response is thought to play the more important role as infected cells are effectively killed by cytotoxic T lymphocytes (CTL), recognizing fragments of viral antigens on the cell surface. These viral antigens are presented in the context of the major histocompatibility complex class I (MHC I) molecules, which are normally present on all nucleated cells. Only cells that express major histocompatibility complex class II (MHC II) molecules on the membrane can function as antigen-presenting cells (APC), which stimulate T helper cells. Activation of T-cells requires two signals; the viral antigen presented in the context of the MHC II, and a costimulatory factor from the APC, notably interleukin (IL) 1. T helper cells can either function to support the CTL (Th1 response), or support the humoral immune response (Th2 response)[173,174]. Th1 clones produce cytokines a.o. IL-2, IFN-gamma and tumour necrosis factor (TNF) beta, whereas Th2 clones produce a.o. IL-4, IL-5, IL-6 and IL-10[143].

Humoral immune response to viruses

The humoral immune response is mediated by B-cells that are primed by contact with a foreign antigen. If the B-cell contains a recognition site on the surface for the antigen, this will lead to clonal proliferation and antibody synthesis. Although the cell-mediated immune response is the best defense in the case of viral infection, there are two points in the infection where antibodies can play a role; firstly, in preventing spread of infection through virus neutralization, and secondly in antibody-dependent cell-mediated cytotoxicity (ADCC), in which antibodies direct NK cells to the appropriate virus-infected target cells.

1.3.2. Immune response to human papillomavirus

The natural HPV infection at anogenital surfaces is poorly immunogenic[84,120], which is probably a reflection of the lack of lytic infection, releasing viral antigens, the absence of a viraemic phase, and also of the limitations of keratinocytes as antigen-presenting cells. HPV has evolved to evade the human immune response by its dependence on these poor APC.

Patients with pre-malignant and malignant anogenital lesions have normal numbers of circulating NK cells, however, *in vitro* these cells show a significantly decreased binding to and killing of HPV-infected cells[129,175]. *In vitro* experiments have shown that the resistance to NK cell mediated lysis is associated with expression of HPV 16 or 18 E7 protein[178].

Downregulation of viral mRNA levels has been shown to be mediated by IFN-alpha, IFN-beta and IFN-gamma[150]. Furthermore, IFN-gamma was shown to reduce HPV-DNA synthesis in monolayer cultures and to inhibit growth in organotypic raft culture[48]. These findings have provided a rationale for the investigation of treatment of precursor lesions (CIN) with interferon. Most information is available on IFN-alpha which appears to be most efficacious in studies in which all visible lesions are surgically removed with subsequent local administration of IFN[33].

Immunosuppressed patients show an increased incidence of HPV infection, CIN lesions and cervical carcinoma, suggesting that cellular immune recognition is essential in elimination of virus-infected cells. In the case of HPV associated invasive cervical cancer, downregulation of MHC class I expression was shown in a large proportion of the tumours[36,39,218] (30-77%), whereas downregulation of MHC class I occurs only rarely in precancer lesions[76,218]. In monomorphic MHC class I downregulated tumours a significant reduction in tumour-infiltrating CD8+ T cells was observed[87]. Furthermore, it has been shown that metastasised tumour cells show a significant decrease in MHC class I expression compared to cells in the primary tumour[40]. This downregulation will have a direct effect on the possibility of the CTL to attack the cancer cells, and suggests a selection of MHC class I negative cells during tumour progression. No correlation could be established between the presence of specific HPV types and MHC class I expression patterns in either CIN or cervical cancer patients[39]. Recently it was also shown that in human keratinocytes in culture, the alterations in MHC class I expression were independent of the presence and amount of E7-specific transcripts[91].

In contrast to MHC class I, MHC class II, which is not normally expressed on squamous cervical epithelium, is upregulated in 80% of cervical squamous cell carcinomas[39]. Upregulation of MHC class II expression in keratinocytes may lead to a state of non-responsiveness or anergy of T-cells[5,71], possibly due to lack of co-stimulatory signals. Genetic variation at HLA class II loci has been investigated both by serologic typing as well as PCR amplification, with controversial results. Some groups have shown an increased risk for carcinoma of the cervix for patients with HLA-DQ3[79,86,229], but this could not be confirmed by other groups[2,75]. Whilst the consequences of upregulation of MHC class II remain unclear, downregulation of MHC class I would suggest that therapeutic immunization against HPV oncogenic proteins may not be effective in targeting CTL to the tumour cells. Nevertheless, some work has been done on both T-helper and CTL epitopes on several proteins from different HPV types. T-helper cell epitopes have been reported on

the HPV type 18 E2 protein[125], the HPV type 1 E4 protein [205] and the HPV type 16 E7 protein[35,190,213]. Cytotoxic T-cell epitopes have been detected on HPV 16 L1 [45,238], E6 [70] and E7[9,64,103,179]. Insertion of the E7 T helper cell epitope in the hepatitis B core antigen as well as conjugation of the peptide to the immunostimulatory carrier protein TraT induced specific T-helper responses in immunized mice[214,215]. CTL could be induced by recombinant vaccinia virus expressing HPV type 16 L1 under an early promotor[238]. Immunisation of peptide alone gives rise to CTL which can eradicate HPV 16 induced tumours in mice[65]. A therapeutic approach to existing HPV infection should target cell-mediated immunity to early HPV proteins in infected epithelium. Self-renewing suprabasal stem cells must be targeted as well as distal, differentiating keratinocytes. Obligatory persistence of E6 and E7 oncoproteins in neoplastic cells permits a vaccine approach directed to these viral tumour-associated antigens[216]. Phase I clinical trials of therapeutic cervical cancer vaccines in humans are currently investigated. For these trials different vaccination strategies are used; a recombinant vaccinia virus vector expressing HPV E6 and E7[20], a glutathione-S-transferase HPV-16 E7 bacterial fusion protein, and two peptides containing HPV-16 E6 and E7 CTL epitopes in combination with a helper peptide[169]. Although the outcomes of these small studies seem encouraging, better results may be anticipated when the vaccines are tested in early stage patients, with a more competent immune system. Recently, the linkage of a sorting signal of the lysosomal-associated membrane protein LAMP-1 to the HPV-16 E7 protein, targetted this protein into the endosomal and lysosomal compartments. This resulted in enhanced presentation to CD4+ T-cells *in vitro*[233]. Treatment with an HPV-16 E7 protein combined with LAMP-1 sorting signal in a vaccinia vector prevented tumour growth in mice after challenge with tumour cells. Furthermore, mice with a small established tumour were cured after vaccination. These results indicate that the sorting signal can improve the *in vivo* therapeutic potency of recombinant vaccines[127].

Study of the humoral immune response to HPV started as long ago as 1965, when Almeida and Goffe[1] used pooled skin wart virions to detect antibodies to papillomavirus antigens in human serum by immunodiffusion. As papillomaviruses do not replicate in tissue culture systems, evaluation of the antibody responses to the genital HPV types has been hampered by the lack of readily available sources of viral proteins for use as target antigens in serological assays. Early studies used disrupted BPV virions to detect antibodies to the papilloma virus group-specific antigen[3,55]. The advance of the recombinant DNA technology has facilitated the production of HPV antigens in large quantities. This, and the use of synthetic peptides, opened the way to epitope mapping of the late[12,53,144] and

early[54,117,144,189,207,212] ORF of low and high risk genital HPV types. Initially, bacterially-derived fusion proteins as well as synthetic peptides containing B-cell epitopes have been used to detect antibodies to various HPV types. In the case of HPV 6, the predominant reactivity was directed to the L1 protein, with occasional reactivities to the L2 and E2 proteins, and no reactivity to any of the other early proteins[95]. In contrast, for HPV type 16 the predominant reactivity was to the L2 protein[96]. The antibody prevalence was not significantly different between adult patients and healthy children[25,96]. However, other studies did observe a statistically significant difference in antibody prevalence between patients (cancer patients or CIN patients) and control groups for the early proteins E2[52,124], E4[98,101,113,155], E6[72,182] and E7[16,97,98,101,113,117,153,182] . Nevertheless, the use of bacterial fusion proteins has some inherent problems: fusion proteins are mostly insoluble, which makes purification difficult. The use of fusion proteins for antibody detection via immunoblotting is time-consuming, leads to denaturation of the protein and will, therefore, not detect antibodies directed against conformational epitopes. Finally, the fusion proteins are generally not pure enough for use in ELISA. Synthetic peptides, on the other hand, are easily obtainable, of high purity, and can be used in ELISA, but will, due to the size of the peptide, only represent linear epitopes, and therefore not detect antibodies to conformational epitopes. This realisation has led to the development of assays incorporating native proteins, both for the early and the late proteins. Late proteins have been shown to self-assemble in virus-like particles, in a variety of expression systems[85,109,119,176,183,237]. Early proteins have been produced in the baculovirus expression system[203,204], and in *in vitro* transcription/translation assays[61,230]. The use of these native proteins in serological assays has led to a significant increase in both sensitivity and specificity[145,227]. Neutralizing antibodies to HPV virions have been demonstrated in humans[28]. In humans, as well as in animal models, neutralizing antibodies are directed to conformational epitopes of the structural proteins[30,89]. Such epitopes are generally type-specific and can discriminate between genotypically closely related HPV types such as HPV-6 and HPV-11[29]. Ideally, prophylactic vaccination should establish a secretory IgA-mediated immunological barrier in the anogenital tract to the viral capsid proteins of the oncogenic HPV types. The long period between vaccination and end-point (up to 20 years), however, make prophylactic vaccination unattractive for cervical carcinoma. Furthermore, even the most efficacious neutralizing antibody response is unlikely to result in full sterile immunity. Once the epithelial cell is infected, the HPV virions will be safe from antibody interference[216]. The antibody response to the early proteins E6 and E7 is unlikely to result in protective

immunity. However, antibodies against these proteins may well have a value as a diagnostic and/or prognostic parameter.

1.4. Aims of the study and outline of this thesis

Recent years have shown a growing interest in serological detection of HPV infection to investigate the possible use in epidemiology and as a diagnostic or prognostic marker for cervical cancer.

Several studies[16,72,97,98,101,113,117,153,182] have shown a significantly higher prevalence of antibodies against transforming proteins E6 and E7 of HPV-16 in cervical cancer patients in comparison with control groups and patients with CIN lesions. However, few data are available on the correlation of antibody prevalence to clinico-pathological indices, such as tumour volume, infiltration depth, differentiation grade, vascular invasion and lymph node metastasis.

The aim of the present study was:

- to study the prevalence of antibodies against the transforming proteins E6 and E7 in cervical cancer patients in comparison with age- and sex-matched controls and patients with CIN lesions;
- to compare the sensitivity and specificity of detection of antibodies against HPV-16 E6 and E7 for the presence of cervical cancer in different assay systems;
- to investigate the relationship between the presence of antibodies against HPV-16 E6 and E7 in pretreatment sera from cervical cancer patients and clinicopathological data, as well as the clinical outcome of the patient;
- to investigate the antibody response against HPV-16 E6 and E7 in cervical cancer patients during and after treatment and during follow-up;
- to investigate the type specificity of HPV 16 E6 and E7 antibody detection;
- to investigate the presence of HPV-16 DNA in histologically negative lymph nodes from cervical cancer patients.

In chapter 2 the prevalence of antibodies against HPV-16 E7 as determined by synthetic peptide ELISA was studied in pretreatment sera from cervical cancer patients and compared to controls and CIN patients. In addition, the presence of these antibodies in pretreatment sera from cervical cancer patients was related to the clinicopathological data and the clinical outcome.

In **chapter 3** the antibody response against HPV-16 E7 in cervical cancer patients, as determined by synthetic peptide ELISA, was investigated during and after treatment and during follow-up.

To relate the presence of antibodies against HPV-16 E6 and E7 to the virus type present in the primary tumour, paraffin-embedded tissue of a number of cervical cancer patients was investigated for the presence and type of HPV by PCR. The optimisation of the PCR to detect high risk types of HPV in paraffin-embedded tissue is described in **chapter 4**.

In **chapter 5** results are presented of the detection of antibodies against HPV 16 E6 and E7 in pretreatment sera from cervical cancer patients, CIN patients and controls by radioimmuno precipitation assay of *in vitro* transcribed and translated E6 and E7 (TT-RIPA). The presence of antibodies against HPV-16 E6 and E7, as detected by synthetic peptide ELISA and/or RIPA, was related to the type of HPV present in the primary tumour. In addition, the presence of antibodies against E6 and E7 as detected by RIPA was related to the clinicopathological data and the clinical outcome.

In **chapter 6** the antibody response against HPV-16 E6 and E7 as determined by RIPA was investigated during and after treatment and during follow-up in cervical cancer patients with HPV type 16 in their primary tumour.

Lymph node metastasis is a strong marker for poor prognosis in cervical cancer. However, approximately 50% of cervical cancer patients with histologically normal lymph nodes and clear resection margins suffer from recurrence after radical surgery. In **chapter 7** the presence of HPV-16 DNA was investigated in histologically negative lymph nodes from cervical cancer patients with HPV type 16 in their primary tumour.

In **chapter 8** the significance of the findings described in this thesis is discussed in relation to studies by other groups.

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ANTIBODIES TO HUMAN PAPILLOMAVIRUS TYPE 16 E7 RELATED TO CLINICOPATHOLOGICAL DATA IN PATIENTS WITH CERVICAL CARCINOMA

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ABSTRACT

Aims-To investigate the correlation between antibodies to the transforming protein E7 of HPV type 16 and clinico-pathological parameters in women with cervical squamous carcinoma.

Methods-A synthetic peptide of the human papillomavirus type 16 E7 protein (amino acids 6 to 35) was used to screen sera from 29 children, 130 women with cervical intra-epithelial neoplasia, 443 women with cervical cancer, and 222 controls for antibodies against human papillomavirus type 16 E7. Bivariate and multivariate analyses were used to investigate the correlation between the serological status in the pretreatment sera and clinico-pathological parameters (the size of the lesion, histological grade, stromal infiltration, vascular invasion, and nodal spread). Furthermore, survival analysis was done using Cox' regression model for all FIGO stages and Stages IB and IIA.

Results-Cervical carcinoma patients had a significantly higher prevalence rate of antibodies to synthetic peptide E7\6-35 than women with cervical intra-epithelial neoplasia (17.7% vs 7%, $p < 0.005$) or controls (17.7% vs 11%, $p < 0.05$). Bivariate analysis of the data on the presence of anti-E7\6-35 antibodies in the pretreatment sera from these patients and clinico-pathological parameters revealed a significant correlation between the presence of anti-E7\6-35 antibodies and the size of the lesion ($p = 0.0009$), histological grade ($p = 0.031$) and lymph node metastasis ($p = 0.011$). In addition, the Cox' regression model, analyzing 4 risk factors which can be determined before treatment, revealed a significant correlation between the presence of anti-E7\6-35 antibodies and a worse prognosis ($p = 0.003$). Survival analysis revealed that both for all FIGO stages ($p = 0.0005$) or Stages IB and IIA alone ($p = 0.0021$) anti-E7\6-35 positive patients before treatment had a significantly shorter life expectancy.

Conclusions-the results of this study show that the presence of antibodies against E7\6-35 in pretreatment sera from patients with cervical carcinoma correlates with the size of the lesion, lymph node involvement and a worse prognosis.

INTRODUCTION

Human papillomaviruses (HPV) are a group of viruses which are associated with various proliferative diseases in the infected epithelium[1]. To date, more than 70 HPV types have been reported. Some of these, among which HPV types 6, 11, 16 and 18 are the most common, are associated with lesions in the anogenital tract[2]. The 'benign' types 6 and 11 are mainly associated with condylomata acuminata. Of the oncogenic HPV types, HPV-16 is most frequently found in cervical carcinomas and the viral genome is often found integrated into the cellular genome[3]. The nuclear protein E7 of HPV-16 is consideredto be one of the two major proteins involved in malignant transformation and maintenance of the transformed phenotype of cells. The viral oncoprotein E7 is consistently transcribed in both HPV positive cervical cancer cell lines and cervical cancers[4,5].

Until recently, few data were available on the humoral immune response to HPV-16. Jochmus-Kudielka *et al*[6] have reported a 14-fold higher prevalence of antibodies to HPV-16 E7 in sera from cervical carcinoma patients than in age and sex matched controls, when fusion proteins were used in western blotting assays for the screening of sera for the presence of HPV antigen specific antibodies. Using synthetic peptides with known B-cell epitopes,[7-9] similar results have been obtained to those found in Western blotting by Jochmus-Kudielka *et al*[6].

To date, no data have been published concerning the prevalence of antibodies against HPV-16 E7 in sera from patients with cervical squamous cell carcinoma in relation to tumour indices, such as lesion size and nodal spread, or to survival. To investigate the possible correlation between the presence of antibodies against HPV-16 E7 and tumour indices, pretreatment sera from cervical carcinoma patients were tested in an enzyme linked immuno-sorbent assay (ELISA) using a synthetic peptide. This peptide was chosen to represent reported major B cell epitopes of HPV-16 E7 (aa 2-21,[10] aa 1-20 and aa 10-30,[7] aa 10-15,[11] aa 10-14,[12] and aa 10-20[13]).

Using bivariate and logistic regression analysis the results obtained for these cervical cancer patients were correlated with the size of the lesion, histological grade, stromal infiltration, vascular invasion, and nodal spread. In addition, anti-E7 positivity was related to survival.

METHODS

SERA

The age distribution of patient and control groups is shown in Table 1. Twenty nine sera of children between 0.5 and 9 years of age were used to determine the cut-off value. One hundred and thirty sera from women with different grades of cervical intraepithelial neoplasia (CIN) and 51 sera from patients with squamous cell cervical carcinoma were obtained from the Department of Gynaecology, University of Amsterdam (head: Prof Dr FB Lammes). A further 392 pretreatment sera from women with squamous cell cervical carcinoma were obtained from the Department of Obstetrics and Gynaecology, University Hospital Groningen. The control group consisted of 222 healthy women with a similar age distribution. No data of HPV DNA typing were available. All women in the Amsterdam control group had normal cytology and were matched to the cancer patient group for age, sex and socioeconomic status. The control group for cervical cancer patients from Groningen consisted of sera sent in for routine screening for other infectious diseases. No data on cytology were available from the Groningen control group.

Table 1: Age distribution of patient and control groups.

	n	Mean Age (SD) (years)
Children	29	3.6 ± 2.5
CIN 1-3	130	32.5 ± 7.9
SCC	443	50.9 ± 15.7
- SCC Amsterdam	51	52.5 ± 13.0
- SCC Groningen	392	50.7 ± 16.0
All Controls	222	50.2 ± 16.3
- Controls Amsterdam	59	51.2 ± 12.8
- Controls Groningen	163	49.3 ± 17.7

CIN = cervical intraepithelial neoplasia; SCC = squamous cell carcinoma

CLINICOPATHOLOGICAL INDICES IN PATIENTS WITH CERVICAL CARCINOMA

The staging of the 443 patients with cervical carcinoma was in accordance with the recommendations of the International Federation of Gynaecology and Obstetrics (FIGO). Clinicopathological data other than stage were only available from 300 patients treated in Groningen. Patients from whom blood samples had been drawn after exconization, were excluded from the analysis. Examination of the patients was performed under general anaesthesia. During this procedure, the lesion size (largest diameter) was estimated

routinely and expressed in centimetres for the large majority of patients.

Patients with stage IB or IIA were mainly treated by preoperative intracavitary radiotherapy, followed by radical hysterectomy with bilateral salpingo-oophorectomy and pelvic lymphadenectomy four weeks later. Postoperative whole pelvis radiotherapy was applied if lymph node metastases or positive resection margins, or both, were present. Patients with stage IIB and most of the patients with stage III were treated by radiotherapy only, if possible followed by adjunctive hysterectomy. In the remaining stage III patients and in all stage IV patients, treatment was individualised using combinations of radiotherapy, surgery, and/or chemotherapy.

To correlate anti-E7 positivity with the histopathological data in bivariate analysis, all the available biopsy or cone material of 300 patients was carefully reviewed. Tumours were classified into well differentiated (grade 1), moderately differentiated (grade 2), poorly differentiated (grade 3) or undifferentiated (grade 4) squamous cell carcinoma, in accordance with the criteria laid down by Ferenczy and Winkler[14].

Criteria for the assessment of the clinical course of the disease were described previously[15]. Briefly, "complete remission" (no evidence of disease) was defined as the absence of all tumour lesions three months after completion of treatment or during follow-up. "Residual disease" existed when tumour lesions were still known to be present after treatment. "Progression of disease" was defined as the appearance of new lesions, the growth of a pelvic mass or other known tumour lesions, or a distinct deterioration in the clinical status of a patient with a known tumour ultimately resulting in death. "Recurrent disease" was used to indicate the reappearance of disease in patients who experienced complete remission. "Poor prognosis" is used to indicate the risk for residual, progressive or recurrent disease. From 367 patients with cervical carcinoma, consecutively admitted and treated at the department of Gynaecological Oncology in Groningen, follow up data were available. Of these 367 patients 106 patients died of cancer.

SYNTHETIC PEPTIDE

The synthetic peptide, encompassing amino acids 6-35 (PTLHEYMLDLQPETTDL-YCYEQLNDSSEEE) of the HPV-16 E7 ORF, was purchased from the Department of Immunology and Medical Microbiology, TNO Medical Biological Laboratory, Rijswijk, The Netherlands. The peptide was synthesized by solid phase synthesis according to Merrifield[16] on polystyrene resin (1 % cross-linking), using an automated peptide synthesiser (SAM-2, Biosearch). Synthetic peptides were purified by chromatography on

Sephadex G-15 and reverse phase high performance liquid chromatography (HPLC).

ELISA

Synthetic peptide (100 μl per well at 5 μg/ml in phosphate buffered saline (PBS), pH 7.2) was coated onto 96 well Maxisorp plates (Nunc) for one hour at 37°C. Control wells were treated in a similar way, with the omission of synthetic peptide from the buffer. After six rinses with distilled water the plates were blocked with 1% gelatin in PBST (PBS containing 0.05% Tween 20) for one hour at 37°C. Sera were incubated in a 1:100 dilution in PBST + 1% gelatin for one hour at 37°C. Goat anti-human IgG peroxidase (1:6700 in PBST, 1% gelatin, Diagnostic Centre SSDZ) was used as a conjugate. After one hour at 37°C the plates were washed six times (PBST), after which substrate was added (3,3',5,5'-tetramethyl-benzidine, Aldrich Chemie). After 20 min the reaction was stopped with 2N H_2SO_4 and colour development read with a Titertek Plate reader at 450 nm.

Each sample was tested in at least three independent experiments. Sera which were eventually scored as equivocal were tested in at least five independent experiments. In each experiment all sera were tested in duplicate on peptide coated wells and control wells, and the mean reactivity of the control wells was subtracted from the mean reactivity of the coated wells. A cut off value was determined from the children's sera using the mean absorption + 3 times the standard deviation as described by Krchnak *et al*[7]. For intratest comparison, positive, negative and borderline positive sera were included in each plate. For intertest comparison, optical densities were recalculated into ratios by division by the cut-off value. Using the cut-off values none of the children's sera was found positive for antibodies against synthetic peptide E7\6-35.

STATISTICAL METHODS

The relationship between clinical stage of the tumour and the presence of anti-HPV-16 E7 antibodies was calculated using the Pearson X^2 test to compare the frequency of the presence of antibodies between 2 or > 2 groups. The interrelationships between anti-E7\6-35 positivity in pretreatment sera from cervical carcinoma patients and tumour related variables were investigated using bivariate and logistic regression analysis. The prognostic value of anti-E7\6-35 and other possible risk factors was determined in Cox regression model. The data used for bivariate and logistic regression analysis of the Groningen patient group included stage of disease (FIGO stage), lesion size, tumour grade, lymph node status, vascular invasion, depth of infiltration and presence of antibodies against E7 synthetic

peptide 6-35. Differences between survival curves were computed by means of the log rank test.

RESULTS

PREVALENCE OF ANTI-E7\6-35 ANTIBODIES IN PATIENT GROUPS AND CONTROLS

The seroreactivity of the different groups to synthetic peptide E7\6-35 is shown in table 2. Twenty-four sera, 19 from patients with cervical carcinoma, one from a CIN patient and four from controls matched to the cervical carcinoma patients, did not give consistent results on repeated testing. These sera, which gave both low positive and high negative results, were scored as equivocal and excluded from statistical analyses.

With peptide E7\6-35, seroreactivity was significantly higher in patients with invasive carcinoma than in the group of matched controls ($p < 0.05$), or women with CIN ($p < 0.005$). No significant differences were found for seroreactivity against peptide E7\6-35 between patients with different grades of CIN (CIN 1-3; data not shown).

Table 2. Prevalence of antibodies against HPV-16 E7\6-35 in sera from patients with cervical intraepithelial neoplasia (CIN), cervical cancer, and controls.

	Anti-E7\6-35 positive			
	N	n	%	Mean ratio (SD)
CIN	129	9[a]	7.0	0.32 ± 0.82
Cervical carcinoma	424	75	17.7	0.61 ± 1.11
Controls	218	24[b]	11.0	0.40 ± 1.02

[a] $p < 0.005$ and [b] $p < 0.05$ when group is compared with cervical carcinoma patients.

PREVALENCE OF ANTI-E7\6-35 ANTIBODIES IN DIFFERENT FIGO STAGES

Prevalence rates of antibodies against peptide E7\6-35 in the different FIGO stages of patients with invasive carcinoma are shown in Table 3. When seroreactivity against peptide E7\6-35 was compared between the four FIGO stages, a statistically significant trend of increasing seropositivity with increasing stage was obtained ($X^2 = 6.38$; $df = 1$; $p = 0.01$). Analysis of the IgG content in patients' sera did not show a statistically significant difference between the different FIGO stages.

Table 3. Prevalence of antibodies against HPV-16 E7\6-35 in sera from 424 patients according to the stage of disease

		anti-E7\6-35 positive	
FIGO stage	N	n	%
IB	203	30	14.6
II	163	30	18.4
III	39	5	12.8
IV	19	10	52.6

CORRELATION OF ANTI-E7\6-35 IN PRETREATMENT SERA FROM PATIENTS WITH CERVICAL CARCINOMA WITH CLINICO-PATHOLOGICAL INDICES

We examined whether tumour associated factors are related to the E7\6-35 serum levels. The results are shown in table 4.Before treatment patients with a lesion size ≥ 4 cm had a significantly higher prevalence of antibodies against E7\6-35 than patients with a lesion size < 4 cm ($p = 0.0009$). In addition, patients with poorly or undifferentiated carcinoma or with lymph node metastases showed a significantly higher prevalence rate of antibodies against E7\6-35 compared to patients with well or moderately differentiated squamous cell carcinoma ($p = 0.031$) or without lymph node metastases ($p = 0.011$). Finally, for patients with vascular invasion of the tumour a significantly higher prevalence rate of anti-E7\6-35 was found in comparison with patients without vascular invasion ($p = 0.04$). In the analysis of clinico-pathological data on anti-E7\6-35 using logistic regression analysis we considered stage, lesion size, differentiation grade, and presence or absence of lymph node metastases. The analysis revealed a significant independent effect of both a lesion size ≥ 4 cm and nodal spread on anti-E7\6-35 positivity ($X^2 = 17.64$; $df = 2$; $p = 0.0001$; sensitivity 33.3%; specificity 90.2%).

We further analysed the predictive value of 4 pretreatment indices (stage, lesion size, differentiation grade, and anti-E7\6-35 positivity) for the presence of lymph node metastases. Logistic regression analysis showed a statistically significant effect of both anti-E7\6-35 positivity and lesion size before treatment on nodal spread ($X^2 = 13.01$; $df = 2$; $p = 0.0015$; sensitivity 28.2%; specificity 90.5%).

Table 4. Pretreatment sera from 300 patients with cervical carcinoma: Bivariate correlation of anti-E7\6-35 positivity with clinico-pathological tumour parameters.

	ANTI-E7\6-35 POS		
	n/total evaluable	%	p[a]
Lesion size			
< 4 cm	14/116	12.1	
≥ 4 cm	40/132	30.3	0.0009
Differentiation grade			
1,2	35/213	16.4	
3,4	24/71	33.8	0.031
Depth of stromal infiltration			
≤ 5 mm	6/32	18.8	
> 5 mm	22/87	25.3	N.S.[b]
Vascular invasion			
Negative	30/177	16.9	
Positive	24/83	28.9	0.04
Lymph node status			
Negative	22/139	15.8	
Positive	16/46	34.8	0.011

[a] X^2 test; [b] N.S.: not significant

ANTI-E7\6-35 ANTIBODIES AND PROGNOSIS

When the presence of antibodies against E7\6-35 in pretreatment sera was correlated with prognosis, a significantly higher prevalence rate of antibodies against E7\6-35 was found in patients who ultimately died of disease (32/105, 30.5%) in comparison with patients who showed no evidence of disease at the closing date of the study (35/243, 14.4%, p = 0.0008).

We performed an analysis of data of 144 patients with Stage IB or IIA. Using Cox regression model we considered three pretreatment prognostic factors (i.e. age, lesion size, and histological grade) which can be determined before surgery, and E7\6-35 positivity (since the lymph node status cannot be determined before surgery, these data were not included). The analysis revealed independent effects of anti-E7\6-35 positivity (p = 0.01)

and - to a lesser extent - lesion size (p = 0.07) on survival. Survival curves in relation to anti-E7\6-35 positivity are shown in the figure. Statistical analysis of the survival curves (log rank test) showed that seropositive stage IB and IIA patients have a significantly shorter life expectancy than seronegative patients (p=0.0021). A similar difference in survival was found for all stages taken together (p=0.0005).

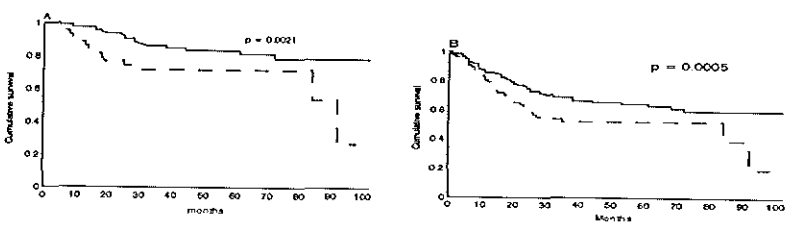

Figure 1. Cumulative survival in anti E7\6-35 negative and positive cervical carcinoma patients.

Legends:
a: FIGO stage IB/IIA patients b: All FIGO stages
— = anti-E7\6-35 negative patients
- - - = anti-E7\6-35 positive patients
X-axis = months of follow up, Y-axis = cumulative survival
The p value of the log rank test is given in the figure.

DISCUSSION

Up to now, no data have been available on the correlation between antibodies against HPV-16 E7 in pretreatment sera from patients with cervical carcinoma and tumour or patient related variables.

An overall prevalence rate of antibodies against peptide E7\6-35 of 17.7% was found in patients with cervical cancer, which was significantly higher than in the control group or CIN patients. Similar results have been reported earlier. Jochmus-Kudielka *et al*[6], using a

bacterial HPV-16 E7 fusion protein, reported a prevalence rate of 20.5% in cervical cancer patients. Krchnak *et al*[7], using a synthetic peptide, comprising amino acids 11 to 30, obtained a prevalence rate of 24%. Müller *et al*[9], using a peptide identical to peptide E7\6-35 in this study, found a prevalence rate of 37% in HPV-16 DNA positive cervical cancer patients. Unfortunately, no data concerning the presence of HPV DNA in the tumour tissues were available from the cervical cancer patients in this study.

When cervical cancer patients were classified according to stage, a significantly different prevalence rate of antibodies against E7\6-35 was observed between the four FIGO stages, the highest prevalence being observed in stage IV patients. Further analysis of the data on anti-E7 positivity in relation to clinico-pathological indices revealed a significant correlation between the presence of antibodies against HPV-16 E7\6-35 in pretreatment sera and a larger lesion size, and lymph node metastasis. After logistic regression analysis only lesion size and the node status showed an independent effect on anti-E7 positivity. This latter finding is in agreement with the data from Onda *et al*[17], which show that lymph node and distant metastases are more frequent in antibody positive patients than in antibody negative patients.

Anti-E7 positivity was also shown to provide prognostic information. When the prognostic value of anti-E7 positivity was analysed in addition to three prognostic indices which can be determined before therapy (age, lesion size, and histological grade), only anti-E7 positivity correlated with poor survival. The prognostic value of pretreatment anti-E7 positivity may be explained by the association of anti-E7 positivity and tumour spread to the regional lymph nodes, which is a strong indicator of poor prognosis.

In conclusion, the results of this study show that the presence of antibodies against E7\6-35 in pretreatment sera from patients with cervical carcinoma correlates with the size of the lesion, lymph node involvement, and a worse prognosis. However, since anti-E7\6-35 antibodies are only detected in a minority of patients with cervical cancer, the clinical value of detection of antibodies against E7\6-35 as a tumour or progression marker is low. The use of E7 antigens resembling the native state, possibly in combination with E6 antigens, might lead to assays with greater sensitivity and specificity[11 18 19]. The results obtained in this study warrant further studies on the value of antibodies against E7 as tumour or progression markers using E7 antigens resembling the native state.

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FOLLOW-UP OF ANTIBODY RESPONSES TO HPV-16 E7 IN PATIENTS TREATED FOR CERVICAL CARCINOMA

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SUMMARY

A synthetic peptide comprising amino acids 6-35 of HPV-16 E7 was used in an ELISA to screen sera taken from 31 cervical carcinoma patients. Sera obtained before and during treatment, and in follow-up, were tested for the presence of antibodies to this peptide. Sixteen patients with negative pretreatment serum determination remained negative during treatment and follow-up. Of the 15 patients with positive pretreatment sera, 12 showed a decrease in anti-E7\6-35 antibody level during treatment. During follow-up an increase in anti-E7\6-35 antibody level was observed in 6 out of 7 patients with progressive or recurrent disease, whereas all patients who remained in complete remission showed stable or further decreasing antibody levels. During the course of disease of the 15 seropositive patients, serum anti-E7\6-35 antibody levels were compared with serum squamous cell carcinoma antigen (SCC-Ag) profiles, a clinically useful tumor marker in the management of cervical cancer patients. Similar patterns were observed in 10 out of 15 patients. The results of this study suggest that in a subset of cervical cancer patients anti-E7\6-35 antibody response against HPV-16 E7 at least partially depends on the presence of viable tumor lesions, and that to some extent the anti-E7 profile reflects the course of disease.

INTRODUCTION

Human Papillomaviruses (HPVs) are a group of viruses that are associated with various proliferative diseases in the infected epithelium [Pfister, 1984]. HPV-16 is predominantly found in cervical carcinoma, and the viral genome is often integrated into the cellular genome [Cullen et al., 1991]. The nuclear protein E7 of HPV-16 is involved in malignant transformation and maintenance of the transformed state of cells. Both in HPV-positive cervical cancer cell-lines and cervical neoplasias, the viral oncoprotein E7 is consistently transcribed [Yee et al., 1985; Van den Brule et al., 1991; Stoler et al., 1992].

Several groups have reported a significantly higher antibody prevalence to HPV-16 E7 in cervical carcinoma patients than in controls [Jochmus-Kudielka et al., 1989; Krchnak et al., 1990; Mann et al., 1990; Müller et al., 1992]. So far, however, no data are available on the effect of treatment on the humoral immune response to HPV-16 E7. Lehtinen et al. [1992] have described the postoperative follow-up of antibody responses to a HPV-16 E2 peptide in cervical carcinoma patients. Their results are suggestive of the existence of a correlation between the antibody responses and the clinical course of disease: 9 out of 11 patients with Stage IB showed decreased posttreatment antibody levels, whereas 4 out of 16 stage III or IV patients initially showed a decrease followed by increasing levels with disease progression. Recently, Dillner [1993] reported a decline in anti-E7 antibody titres in cervical carcinoma patients during treatment and follow-up. However, no data on the clinical course of disease were given.

In the present study, in order to investigate the relation with treatment results and/or course of disease, a synthetic peptide (E7\6-35) was used to analyze antibody levels to HPV-16 E7 in sera taken before, during, and after treatment, and during follow-up of 31 women with cervical carcinoma. As reported by Müller et al. [1992] the prevalence of antibodies to this peptide is significantly higher in cervical cancer patients than in controls. The results from this study were compared to the serum levels of squamous cell carcinoma antigen (SCC-Ag), a clinically useful tumor marker in the management of cervical cancer patients [Kota et al., 1979; Duk et al., 1990].

MATERIALS AND METHODS

Patients

The patients were staged according to the recommendations of the International Federation of Gynaecology and Obstetrics (FIGO). Patients were selected to represent all FIGO stages. Nine patients were classified as stage IB, 8 as stage IIA or -B, 8 as stage IIIB, and 6 as stage IVA or -B. The median age of the patients was 44.5 (25.1-74.4) years. All patients had squamous cell carcinoma and were classified according to the criteria of Ferenczy and Winkler [1987]. Since 1987 all patients entered the "Cervical Cancer Data Bank" of the University Hospital Groningen, prospectively. Clinical and histopathological data from patients treated before 1987 were carefully reviewed.

Sera

Serum samples were obtained from 31 women with cervical carcinoma attending the Department of Obstetrics and Gynaecology, University Hospital Groningen, The Netherlands. Pretreatment sera and a median number of 5 sera (range 1-8) during and/or after treatment were taken. The median interval between pretreatment serum and last serum was 340 (21 - 2,298) days.

Treatment

Patients with Stage IB or IIA and a tumor diameter < 4 cm were treated by radical hysterectomy with bilateral salpingo-oophorectomy and pelvic lymphadenectomy. Postoperative radiotherapy was applied if lymph node metastases and/or positive resection margins were present. Patients with bulky tumors (lesion size $\geq$ 4 cm) were treated by a combination of chemotherapy (5-fluorouracil/carbo-platine) and external and intracavitary irradiation, and if possible followed by adjunctive hysterectomy with pelvic and/or paraaortic lymph node sampling (this treatment schedule has been applied since 1987 within the framework of a phase II clinical trial).

Criteria for the judgment of the clinical course of disease have been described previously [Duk et al., 1990]. Briefly, 'complete remission' was defined as the absence of all known tumor lesions, 3 months after treatment or during follow-up. 'Residual disease' existed when tumor lesions were still known to be present after treatment. 'Progressive disease' (either within 3 months after treatment or during follow-up) was defined as the appearance of new lesions, the growth of known tumors, or a distinct deterioration in the clinical status

of a patient with tumor, ultimately resulting in death. 'Recurrent disease' was used to indicate the reappearance of disease in patients who experienced complete remission.

Synthetic peptide

A peptide, comprising amino acids 6-35 of the HPV-16 E7 ORF (PTLHEYMLDLQ-PETTDLYCYEQLNDSSEEE) was purchased from the Department of Immunology and Medical Microbiology, TNO Medical Biological Laboratory, Rijswijk, The Netherlands. The peptide was synthesized by solid-phase synthesis according to Merrifield [1963] on polystyrene resin (1% crosslinking), using an automated peptide synthesizer (SAM-2, Biosearch, San Rafael, CA).

Synthetic peptide ELISA

The synthetic peptide (100 μl per well at 5 μg/ml in PBS, pH 7.2) was coated onto 96-well Maxisorp plates (Nunc, Roskilde, Denmark) for 1 hr at 37°C. The plates were blocked with 1 % gelatin in PBST (PBS containing 0.05 % Tween-20) for 1 hr at 37°C. Sera were diluted 1:100 in PBST and incubated for 1 hr at 37°C. Goat anti-human IgG peroxidase (1:6,700 in PBST, Diagnostic Center SSDZ) was used as a conjugate (1 hr at 37°C), followed by the addition of substrate (3,3',5,5'-tetramethyl-benzidine, TMB, Aldrich Chemie, Bornem, Belgium; 20 min, room temperature). The reaction was stopped with 2N H_2SO_4, and color development read in a Titertek plate reader (Flow Laboratories, The Netherlands) at 450 nm. A cutoff value was obtained from the testing of 29 children's sera on E7\6-35, using the mean absorption plus 3 times the standard deviation. Each sample was tested at least 3 times in separate experiments. For intertest comparison known positive, negative, and borderline positive sera were included in each plate. Results were expressed as a ratio (mean absorbance of patient serum divided by mean absorbance of cut-off serum). Sera showing a ratio higher than 1.0 were considered positive.

Determination of IgG content in sera

IgG content in all sera from patients with cervical carcinoma was determined by ELISA as follows. Goat anti-human IgG (affinity-purified, Zymed, San Francisco, CA) was coated (1μg/ml in carbonate buffer pH 9.6) overnight at 37°C. Blocking was done with 1% BSA in carbonate buffer for 2 hr at 37°C. Dilution series of patient sera starting at 1:10,000 were tested in duplicate. After incubation for 1 hr at 37°C, the plates were washed 6 times with PBST. Peroxidase-labelled goat anti-human IgG (1:6,700, Diagnostic Center SSDZ)

Fig.1a.Antibody levels to E7\6-35 during and after treatment and during follow-up in patients with complete remission following treatment. -□-, patient 6, stage IB, N-[1]1, after internal radiation; 2 , after radical surgery; 3,4, NED[2], 8 and 48 months after treatment. -■-, patient 7, stage IB, N[1] . 1 , after radical surgery; 2,3, NED[2], 7 and 9 months after treatment. -▽-, patient 8, stage IB, [1]N- 1, after internal radiation; 2 , after radical surgery; 3,4,5,6,7,8, NED[2], 8,10,44,56,74, and 86 months after treatment. -●-, patient 11, stage IIA, N-[1].1, during combination chemo/radiotherapy; 2, before surgery; 3 , after surgery; 4,5, NED[2], 14 and 20 months after treatment. -○-, patient no. 13, stage IIA, N?[3].1, start external radiation; 2, after external radition; 3 , after internal radiation; 4,5, NED[2], 5 and 9 months after treatment. -△- patient no. 25, stage IIIB, N?[3]1, during combination chemo/radiotherapy; 2, after combination chemo/radiotherapy; 3,4,5, NED[2], 3, 7, and 19 months after treatment.[1] = node status negative; [2] NED = no evidence of disease; [3] = node status unknown; PT = pre-treatment; underlined number = serum sample drawn approximately 3 months after ending treatment. **b:** Antibody levels to E7\6-35 during and after treatment and during follow-up in patients with residual disease following treatment.-○- patient 10, stage IIB, N?[1]1, start external radiation; 2, after external radiation; 3 ,after 2x internal radiation, residual disease, 4,5, progressive disease, 2 and 3 months later. -●- patient 21,stage IIIB, N?[1]. 1, during external radiation; 2, after external radiation, 3 , residual disease. -□- ,patient 29, stage IVB, N?[1]. 1 , after (palliative) external radiation, 2,3, progressive disease, 3 and 6 months later. -△-, patient 30, stage IVB, N?[1].1, start (palliative) external radiation; 2, progressive disease during radiation. [1] node status unknown; PT = pre-treatment; underlined number = serum sample drawn approximately 3 months after ending treatment. **c:** Antibody levels to E7\6-35 during and after treatment and during follow-up in patients in complete remission following treatment, with reappearance of disease during follow-up. -●- patient 5, stage IB, N-[1]. 1, 3 months after radical surgery; 2, recurrence, 5 months after treatment; 3, progression, 7 months after treatment. -□-, patient 9, stage IB, N+[2]. 1, after internal radiation; 2, after radical surgery; 3 start external radiation; 4, 5, NED[3] 2 and 3 months after treatment; 6, recurrence, 4 months after treatment; 7, progression, 13 months after treatment. -■-, patient 12, stage IIB, N?[4]. 1,2,3, during combination chemo/radiotherapy; 4, NED[3] , 4 months after treatment; 5, recurrence, 7 months after treatment. -○-, patient 16, stage IIB, N-[1]. 1,2, during combination chemo/radiotherapy; 3, before surgery/NED[3]; 4,5, NED[3] , 4 and 8 months after treatment; 6, recurrence, 9 months after treatment. -△-, patient 31, stage IVA, N?[4]. 1,2 during combination chemo/radiotherapy; 3,4, NED[3], 3 and 5 months after treatment; 5, recurrence, 8 months after treatment; 6, progression, 10 months after treatment. [1] = node status negative;[2] = node status positive; [3] NED = no evidence of disease; [4] = node status unknown; PT = pre-treatment; underlined number = serum sample drawn approximately 3 months after ending treatment

Figure 1a

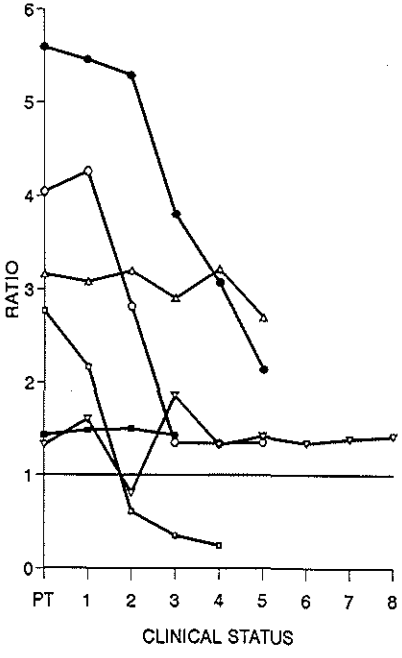


Figure 1b

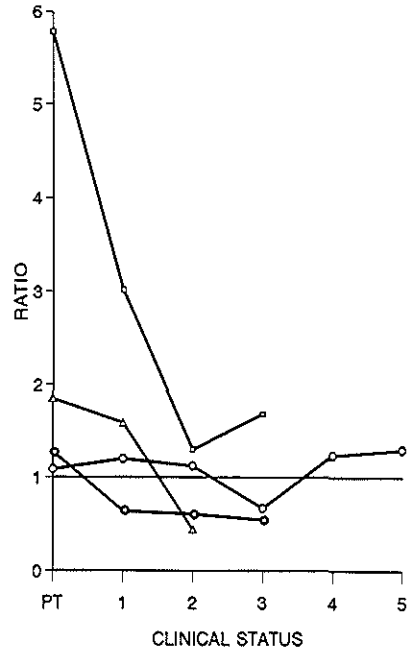
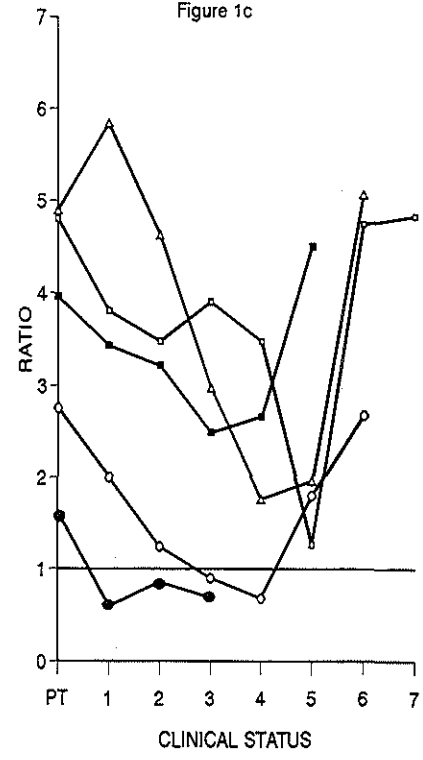


Figure 1c



was used as a conjugate. After 1 hr at 37°C, the plates were washed 6 times, and TMB and peroxide were added to the wells. After 20 mins at room temperature the reaction was stopped by the addition of 2N H_2SO_4, and the absorbance read in a Titertek plate reader at 450 nm. A donor serum, containing 10.7 g/l IgG as determined by nephelometry, was used as reference. This same serum was used to obtain an IgG negative fraction after protein G separation, to be used as a negative control. IgG content of the sera was calculated on basis of the standard curve obtained for the reference serum.

ELISA for IgG antibodies against *Toxoplasma gondii* and Rubella virus

ELISA for detection of IgG antibodies against *Toxoplasma gondii* and rubella virus was performed as described previously [Herbrink et al., 1987]. Briefly, *Toxoplasma* antigen or rubella was coated to the wells of microtiterplates. Following serum incubation and a washing step, *Toxoplasma* or rubella specific IgG antibodies were detected using a peroxidase-conjugated goat anti-human IgG serum.

SCC-Ag

Serum levels of squamous cell carcinoma antigen (SCC-Ag) were determined in a previous study by Duk et al. [1990]. Serum levels of SCC-Ag were measured in a microparticle enzyme immunoassay with 2 different monoclonal antibodies using the IMx analyzer system from Abbott Diagnostics (Chicago, IL). Upper limit of normal was defined as 2.5 ng/ml, being the 95th percentile in female blood donors.

RESULTS

Serum anti-E7\6-35 antibody response and treatment results

Before treatment 15 of the 31 patients demonstrated an anti-E7\6-35 antibody concentration higher than the cut-off value: 5 of 9 patients with stage IB, 5 of 8 patients with stage II, 2 of 8 patients with stage III, and 3 of 6 patients with stage IV.

All patients who were negative for antibodies to E7\6-35 before treatment remained negative during treatment and follow-up.

During treatment a decline in anti-E7\6-35 antibody response was observed in 12 out of the 15 seropositive patients. In 3 patients anti-E7\6-35 antibody levels remained stable, both during and after treatment and during follow-up. Figures 1a-c show the anti-E7\6-35 antibody profiles for the 3 different patient groups: complete remission, residual disease,

and recurrent disease.

To exclude the possibility that the decrease in anti-E7\6-35 antibody level was due to changes in total IgG concentration, the total IgG content of all sera was determined. No significant changes in IgG content were found in individual patients during and after treatment (data not shown). Similarly, no significant changes in IgG antibody titer against rubella and *Toxoplasma gondii* were observed, both during treatment and follow-up.

Serum anti-E7\6-35 antibody levels after treatment and during follow-up

Six weeks to 3 months after the end of therapy, 9 of the 15 patients showed anti-E7\6-35 antibody levels exceeding the threshold value (Fig 1a-c).

Anti-E7\6-35 antibody levels during follow-up were compared with anti-E7\6-35 antibody levels after completion of therapy. The results are shown in Table 1. From 2 patients, due to rapid progression of disease, no follow-up sera were available.

The median follow-up of the 6 patients with no evidence of disease at the closing date of the study was 39 months (range, 16-76 months). Six weeks to 3 months after ending therapy, 5 of these 6 patients showed anti-E7\6-35 antibody levels exceeding the threshold value. None of these patients showed an increase in anti-E7\6-35 antibody level during follow-up.

After completion of therapy, 3 of the 5 patients who finally suffered from recurrence of disease showed anti-E7\6-35 antibody levels exceeding the threshold value. In 4 of these 5 patients an increase in anti-E7\6-35 antibody level during follow-up accompanied the clinical manifestation of the disease. In 2 of these 4 patients the increase in anti-E7\6-35 antibody level preceeded symptomatic tumor activity by 11 and 15 weeks, respectively. All 5 patients died of disease.

Of the 4 patients with residual disease following treatment (Figure 1b), 1 patient had an anti-E7\6-35 antibody level exceeding the threshold value after completion of therapy. From 2 patients, due to rapid progression of disease, no follow-up sera were available. The other 2 patients showed an increase in anti-E7\6-35 antibody level which accompanied progression of the disease. Overall, 6 of 7 patients with recurrence or progression of disease showed an increase in anti-E7\6-35 antibody level.

Table 1. Serum anti-E7\6-35 antibody levels after completion of therapy and during follow-up in relation to the clinical assessment of the disease status.

		Anti-E7\6-35 response	
		No. of patients positive 3 months after treatment	No. of patients with an increase during follow-up
Disease status	N		
NED[1] at end of study	6	5	0
Recurrent disease during follow-up	5	3	4
Residual disease	2	1	2

[1] NED, no evidence of disease

Relation between the serum anti-E7\6-35 antibody response and the serum SCC-Ag level

Anti-E7\6-35 antibody profiles before, during, and after treatment and during follow-up were compared with serum SCC-Ag levels in patient sera; 4 examples are shown in Figure 2a-d. Generally, anti-E7\6-35 antibody levels adjust more slowly than serum SCC-Ag levels both in respect to increase and decrease. Similar patterns of anti-E7\6-35 antibody profiles and serum SCC-Ag levels were obtained for 10 out of 15 patients. In 5 patients anti-E7\6-35 antibody profiles did not correlate with serum SCC-Ag levels. Three of these 5 patients were negative for serum SCC-Ag both before, during and after treatment.

DISCUSSION

To study the anti-E7\6-35 antibody response during treatment and follow-up, serial serum samples from 31 patients with cervical squamous cell carcinoma were studied longitudinally. The sera were analyzed for changes in antibody levels to HPV-16 E7 using a synthetic peptide (E7\6-35). The 31 patients were selected to represent all FIGO stages.

Before treatment, 16 patients had anti-E7\6-35 antibody levels below the cutoff value.

Figure 2a

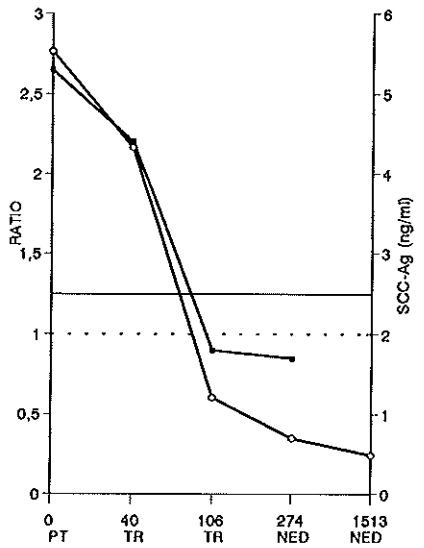


Figure 2b

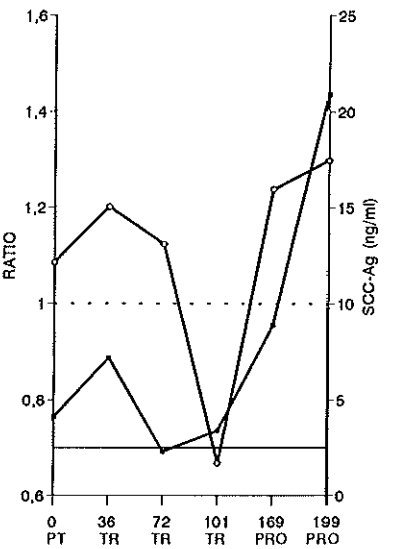


Figure 2c

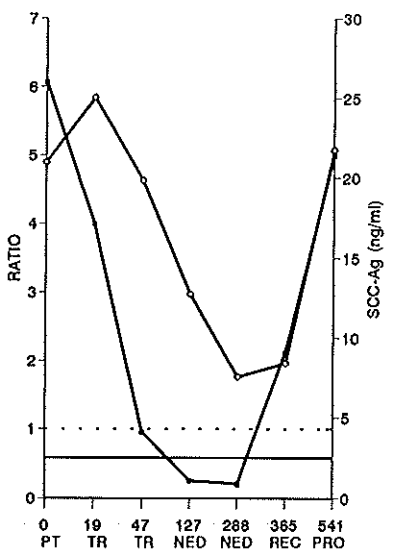


Figure 2d

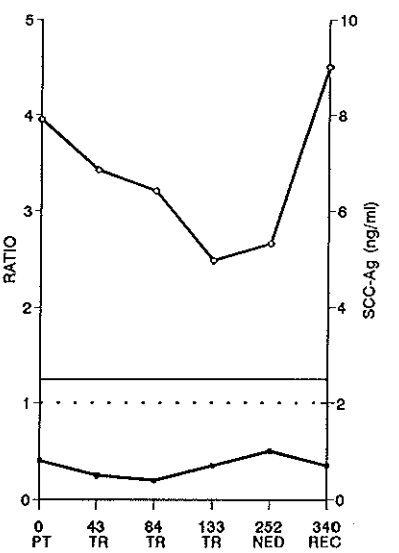


Figure 2. Antibody levels to E7\6-35 before treatment and during treatment and follow-up in comparison to serum SCC-Ag level.
Legends
-o- Anti-E7\6-35 antibody level
-■- Serum SCC-Ag level
..... cut-off value of anti-E7\6-35 antibody level
___ cut-off value of serum SCC-Ag level
X-axis: Number of days after diagnosis and clinical status
Left Y-axis: Ratio in peptide E7\6-35 ELISA
Right Y-axis: Serum SCC-Ag in ng/ml
Figure 2a: patient no. 6, complete remission.
Figure 2b: patient no. 10, residual disease.
Figure 2c: patient no. 31, recurrent disease.
Figure 2d: patient no. 12, recurrent disease.

PT = pre-treatment, TR = treatment, NED = no evidence of disease, REC = recurrence, PRO = progression

None of the sera from these patients taken during or after treatment were positive for antibodies against E7\6-35, suggesting that the HPV-16 E7 epitope-specific immune response is not changed due to treatment. These patients may produce antibodies against other, possibly conformational epitopes, which are not detected in our assay. In addition, the tumor tissues from some of these patients may contain other oncogenic HPV types (e.g. HPV-18, 31 or 33).

Twelve of the remaining 15 patients with positive pretreatment sera, showed a decrease in anti-E7\6-35 level during treatment. This decrease could not be attributed to a decrease in total IgG content in the sera and, consequently, it is considered anti-E7 specific. The decrease in anti-E7 antibody level is therefore suggestive of an association with a reduction in tumor size.

In 3 of the 6 patients with no evidence of disease during follow-up, serum anti-E7\6-35 antibody levels remained at a constant level during and after therapy and during follow-up. Considering the period of complete remission (16 - 76 months), it is unlikely that the elevated levels of anti-E7\6-35 are indicative of the presence of subclinical metastases.

The finding that in 6 of the 7 patients with recurrence or progression of disease,

compared to none of the 6 patients with complete remission, an increase in anti-E7\6-35 antibody level paralleled tumor progression suggests a correlation with tumor load or tumor proliferation rate. This is supported by the observation that a majority of the patients showed a similar pattern of serum trends of anti-E7\6-35 and SCC-antigen, a well-established tumor marker for patients with cervical squamous cell carcinoma [Kato et al., 1979; Duk et al., 1990].

In conclusion, the results from the present study suggest that in a subset of cervical cancer patients, elevated anti-E7\6-35 antibody levels correlate with the tumor load, and that recurrent or progressive disease can be associated with an increase in anti-E7\6-35 antibody level. It is, however, too soon to assess the clinical implications of this observation.

Further improvement of both sensitivity and specificity of monitoring the antibody response to HPV-16 E7 may be achieved by using E7 antigens resembling the native state. Such studies are currently under investigation in our laboratory.

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A COMPREHENSIVE STUDY OF SEVERAL GENERAL AND TYPE-SPECIFIC PRIMER PAIRS FOR DETECTION OF HUMAN PAPILLOMAVIRUS DNA BY POLYMERASE CHAIN REACTION IN PARAFFIN-EMBEDDED CERVICAL CARCINOMAS

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We have compared the efficacy of three general primer pairs for the detection of human papillomavirus (HPV) DNA in formaldehyde-fixed paraffin-embedded carcinomas. The use of these primer pairs leads to underestimates of the HPV prevalence (GP5/6 61.1%; CPI/IIG 57.4%; MY09/11 46.9%; combined 72.8%). The efficacy of each primer pair seemed to be inversely correlated to the length of the amplimer produced. By using newly developed type-specific primer pairs (amplimer length, approximately 100 bp), an increase in HPV DNA detection (87.6%) was found.

There is strong evidence to link the presence of human papillomaviruses (HPV) to the development of cervical cancer (11). The presence of HPV DNA in over 90% of carcinomas of the uterine cervix has been established by PCR (12, 18, 20). Ideally, one primer pair would suffice for PCR amplification of all mucosatropic HPV types (6, 9, 14, 16, 20). As paraffin-embedded material is the main source for retrospective studies using PCR, the efficacies of three general primer systems with archival tissues were investigated, along with those of HPV type-specific primer pairs and sequence analysis.

Paraffin-embedded tissues from 162 women treated for squamous cell cervical carcinoma at the Department of Obstetrics and Gynaecology, University Hospital, Groningen, were available. DNA was extracted from 10-μm sections by deparaffination and digestion with proteinase K as described by Wright and Manos (19). Adjacent sections were hematoxylin and eosin stained and assessed for presence of tumor cells.

All oligonucleotide primers were obtained from Pharmacia, Woerden, The Netherlands. The primers used were the MY09/MY11 set (MY09/11) (9), the GP5-GP6 set (GP5/6) (14), and the CP-I-CP-IIG set (CPI/IIG) (16) (Table 1). As a control for DNA extraction, ß-globin primers PC03 and PC04 (13) were used. Conventional TS primers were used for HPV type 16 (HPV-16), -18, -31 and -33 (4, 10, 18). In addition, for detection of HPV-16, -18, -31 and -33 DNAs, supplementary primer pairs (new TS primers) spanning shorter DNA sequences, were constructed (Table 2). PCR amplification was performed by the method of Saiki et al. (13). The final 100-μl PCR mixture contained 10-μl sample, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin, 200 μM deoxynucleoside triphosphates, 50 pmol of each primer, and 0.25 U of SuperTaq (Sphaero Q, Cambridge, United Kingdom), except for the MY09/11 PCR, in which 0.5 U of SuperTaq was used. The standard PCR conditions were 1 min at 95°C, 1 min at the

TABLE 1. Specification of oligonucleotides used as primers and probes for general HPV detection by PCR

Primer or probe	Sequence (5'-3')[a]	Target[b]	Amplimer length	Reference
Primers				
MY09	CGTCCMARRGGAWACTGATC	L1	452	9
MY11	GCMCAGGGWCATAAYAATGG			
GP5[c]	TTTGTTACTGTGGTAGATAC	L1	155	14
GP6	ACTAAATGTCAAATAAAAAG			
CP-I	TTATCAWATGCCCAYTGTACCAT	E1	188	16
CP-IIG	ATGTTAATWSAGCCWCCAAAATT			
PC03	ACACAACTGTGTTCACTAGC	ß-globin		13
PC04	CAACTTCATCCACGTTCACC			
Probes[d]				
GP1	CTGTTGTTGATACTACACGCAGTAC			17
GP2	CTGTGGTAGATACCACWCGCAGTAC			17
PR-G	AGCAYTRTATTGGTATMGAACAGG			16

[a] M, A + C; R, A + G; S, G + C; W, A + T; Y, C + T.

[b] L1, HPV late structural protein 1; E1, HPV early protein 1.

[c] Used in combination with random prime-labelled probe GP5/6.

[d] GP1 and GP2 are general probes used with MY09/11, and PR-G is a probe for genital HPV used with CPI/IIG.

annealing temperature, and 2 min at 72°C for 40 cycles. After PCR, 25-30 μl of all samples was run on a 2% agarose gel; gels were blotted (Hybond N^+; Amersham, Little Chalfont, United Kingdom) and hybridized with the appropriate probe (Tables 1 and 2). Blots were washed twice for 15 min in 2xSSC (1xSSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate. For all samples positive in general PCR and negative in both TS PCR, the general PCR amplimer bands were sequenced by the protocol of the manufacturer of the T7 sequencing kit (Pharmacia).

All patients were positive in the ß-globin PCR (100-bp DNA fragment). Of the 162 carcinomas, 118 (72.8%) were positive for HPV DNA; 99 (61.1%) were positive with GP5/6, 93 (57.4%) were positive with CPI/IIG, and 76 (46.9%) were positive with MY09/11 (Table 3). All tissue samples were also tested with conventional TS primer pairs. Of 118 carcinomas positive with one or more general primer pairs, 59 carcinomas were positive using conventional TS primers; the remaining 59 carcinomas were still untypable. None of the tissue samples negative with general primers were positive with conventional TS primer pairs. Further evaluation was performed with newly selected TS primer pairs.

By using these new TS primer pairs, 43 of the 59 untypeable carcinomas could be typed. Furthermore, using these new TS primer pairs, 24 of the 44 carcinomas negative with all general HPV primer pairs were positive

TABLE 2. Specification of oligonucleotides used as primers and probes for TS HPV detection by PCR

Primer pair or probe	Sequence(s) (5'-3')[a]	Amplimer length	Ref.
Primer pairs			
Conventional TS 16	GTGTGTACTGCAAGCAACAG, GCAATGTAGGTGTATCTCCA	395	8
New TS 16	GGTCGGTGGACCGGTCGATG, GCAATGTAGGTGTATCTCCA	96	
Conventional TS 18	AAGGATGCTGCACCGGCTGA, CACGCACACGCTTGGCAGGT	217	17
New TS 18	CCTTGGACGTAAATTTTTGG, CACGCACACGCTTGGCAGGT	115	
Conventional TS 31	ATGGTGATGTACACAACACC, GTAGTTGAGGACAACTGAC	514	6
New TS 31	GGGATTGTTACAAAGCTACC, CGCTTAGTAGACGTCGTCGC	110	
Conventional TS 33	ATGATAGATGATGTAACGCC, GCACACTCCATGCGTATCAG	506	17
New TS 33	CCACCACTGCTTCTTACCTC, ACCATTTTCATCAAATGGGA	114	
Probes			
TS 16[b]	CAAGAACACGTAGAGAAACCCAGCTGTAAT		8
TS 18	TGGTTCAGGCTGGATTGCGTCGCAAGCCCA		17
TS 31	ACCTGCGCCTTGGGCACCAGTGAAGGTGTG		6
TS 33	CAAATGCAGGCACAGACTCTAGATGGCCAT		17

[a] For primer pairs, the sequences are given in the order of the 5' primer followed by the 3' primer

[b] The new TS 16 primers were used in combination with random prime-labelled HPV-16 E6 probe.

for HPV (19 contained HPV-16 DNA, and five contained HPV-18), thus raising total HPV positivity to 87.6% for this group of 162 cervical carcinomas. In this way, 95 carcinomas were found to contain HPV-16 DNA, 23 contained HPV-18 DNA, 2 contained HPV-31 DNA, 5 contained HPV-33 DNA, whereas 1 carcinoma was positive for both HPV-16 and -31.

Sixteen carcinomas positive for general primers were still untypeable; of these, 14 were subjected to sequencing. From two patients, insufficient amounts of material were available for sequencing. Other HPV types found in this way were HPV-6, HPV-34 (twice), HPV-45, HPV-52, HPV-58, HPV-X (some similarity to HPV-34 [data not shown]), and HPV-X (some similarity to HPV-18, not reacting with the conventional or new HPV-18-specific primer pair [data not shown]). Five carcinomas were HPV-16 positive, and one was HPV-33 positive, but these did not react with conventional or new TS primers.

The results described above show that the use of one general HPV primer pair with paraffin-embedded tissues grossly underestimates the presence of HPV DNA in the studied

population. Moreover, even the combination of the three most widely used primer pairs, MY09/11, GP5/6 and CPI/IIG, still results in an underestimation of HPV prevalence.

Several authors have reported difficulties in reproducing PCR results with formaldehyde-fixed tissues (1, 2, 5, 15). Recently, the antagonistic effect of formaldehyde fixation due to DNA modification, causing the inhibition of PCR amplification of longer (>200 bp) amplimers, has been described by Karlsen et al. (7).

Our results show that the efficiency of the primer pair is inversely correlated to the length of the amplimer and that the amplification of a PCR product of less than 200 bp is also affected by formaldehyde fixation as the GP5/6 (amplimer length 155 bp; positivity 61.1%) and CPI/IIG (188 bp and 57.4%, respectively) primer pairs do not reach the overall positivity of 87.6%.

TABLE 3. HPV PCR-positive results of 162 cervical carcinomas with three general primer sets.

PCR results with primer set[a]			
GP5/6	CPI/IIG	MY09/11	no. with pattern
-	-	-	44
+	-	-	11
-	+	-	8
-	-	+	4
+	+	-	23
+	-	+	10
-	+	+	7
+	+	+	55

[a]Of 162 carcinomas, 118 (72.8%) were positive with at least one primer set. The total numbers detected by primer sets GP5/6, CPI/IIG, and MY09/11 were 99 (61.1%), 93 (57.4%), and 76 (46.9%), respectively.

The newly designed TS primers proved to be of great value, since 72.9% of the general primer positive untypeable carcinomas could be typed. Furthermore, with the TS new primers, 24 of the 44 general HPV PCR-negative carcinomas were found to contain HPV DNA.

It can be argued, however, that the amplification of these 100-bp amplimers is still hampered by formaldehyde fixation, as studies using fresh tissue samples commonly report HPV prevalences well over 90% (8, 12, 18, 20). Alternatively, the HPVs not detected by the TS primers are types other than HPV-16, -18, -31 and -33.

In conclusion, general primer pairs are unsatisfactory for the amplification of HPV

DNA from formaldehyde-fixed paraffin-embedded tissue samples. Therefore, we propose the following strategy for cervical carcinomas: amplification with TS new primers (giving approximately 100-bp products), amplification of TS-negative tissues with CPI/IIG, GP5/6 and/or MY09/11 primer pairs, and subsequent sequencing to detect other HPV types. At this moment, this should lead to the highest possible detection of HPV DNA from formaldehyde-fixed paraffin-embedded carcinomas.

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RELATION BETWEEN HPV-16 SEROLOGY AND CLINICO-PATHOLOGICAL DATA IN CERVICAL CARCINOMA PATIENTS: PROGNOSTIC VALUE OF ANTI-E6 AND/OR E7 ANTIBODIES

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ABSTRACT

The clinical significance was investigated of the enhanced sensitivity of anti-HPV-16 E6 and E7 antibody detection by radio immunoprecipitation assays (RIPA). This was done using *in vitro* translated HPV-16 E6 and E7 proteins. The data were compared with those obtained using the E7\6-35 synthetic peptide ELISA. The results obtained with E6 and E7 RIPA were related to clinico-pathological data from cervical carcinoma patients positive for HPV type 16 DNA in their primary tumour. The antibody prevalence to E6, E7, E6 and/or E7 and E6 and E7 as determined by RIPA was significantly higher in cervical cancer patients as compared to both controls and CIN patients. Odds ratios, calculated for cervical carcinoma patients versus controls, ranged from 7.4 to 15.4. Antibodies to E6 and/or E7 were largely restricted to patients with HPV DNA in their primary tumour.

Analysis of the relation between prevalence of antibodies to E6 and E7 and clinico-pathological parameters was limited to 85 HPV-16 positive patients. The strongest relation with clinico-pathological data, such as lesion size, lymph node involvement, and prognosis was found for E7 synthetic peptide ELISA, whereas E6 and E7 RIPA did not reach significance. The significance of these findings is discussed.

INTRODUCTION

The strong association between the presence of mucosal human papillomavirus types (predominantly types 16, 18, 31 and 33) and the development of cervical cancer[12] has boosted the interest in human papillomavirus serology, to investigate the possible use in epidemiology and as a diagnostic or prognostic marker. HPV type 16 is most frequently found in squamous cell cervical carcinomas, and the viral oncoproteins E6 and E7 are consistently transcribed in HPV positive cervical cancer cell lines and cervical neoplasias [14,17,22]. Therefore, serology has focused for a large part on these two major transforming proteins of HPV type 16. Using synthetic peptides and fusion proteins several authors have reported a significantly higher antibody prevalence to HPV-16 E6[6,11] and E7 [1,7,9,10,11,13] in cervical carcinoma patients than in controls. Neither synthetic peptides, nor fusion proteins, are ideal antigens in serological assays, as only non-conformational epitopes will be presented. Recently, a number of studies have been performed using native proteins derived from *in vitro* translation[3,11,18-20] or baculovirus expression[15,16]. These studies report increases in both sensitivity and especially specificity compared to serological tests using antigens with non-conformational epitopes only. In order to validate the clinical significance of the enhanced sensitivity of antibody detection by RIPA of native proteins over synthetic peptide ELISA, we have performed RIPA for HPV-16 E6 and E7 and analyzed whether the results relate to clinico-pathological data from cervical carcinoma patients. The findings were then compared to those obtained previously[1], using the synthetic peptide ELISA.

METHODS

Patients and controls.

Pretreatment sera from 392 women with squamous cell cervical carcinoma were obtained from the Department of Obstetrics and Gynaecology, University Hospital Groningen. The mean age of these patients was 50.7 years (SD 16.0 yr). HPV DNA detection has been performed for 137 of the 392 cervical cancer patients on paraffin-embedded tissues as described elsewhere[2]. The control group consisted of 197 healthy women with a similar age distribution (50.2 years SD 16.7). The control group for cervical cancer patients consisted of sera sent in for serology for infectious diseases unrelated to STDs. No data of cytology or HPV DNA typing were available from the control group. In addition, sera from a group of 111 women with cervical intraepithelial neoplasia (CIN) lesions were obtained (age 32.5

years SD 7.9; CIN 1, N = 13; CIN 2, N = 23; CIN 3, N = 75).

Clinico-pathological parameters of patients with cervical carcinoma .

The staging of the 392 patients with cervical carcinoma was in accordance with the recommendations of the International Federation of Gynaecology and Obstetrics (FIGO). Clinico-pathological data other than stage were available from 300 patients. Blood samples drawn after exconization was a criterion for exclusion from the analysis. Examination of the patients was performed under general anaesthesia. During this procedure, the lesion size (largest diameter) was estimated routinely and expressed in centimeters for the large majority of patients.

All the available biopsy or cone material of 300 patients was carefully reviewed. Tumours were classified into well - (grade 1), moderately - (grade 2), poorly - (grade 3) or un-differentiated (grade 4) squamous cell carcinoma, in accordance with the criteria laid down by Ferenczy and Winkler[5].

From 367 patients with cervical carcinoma, consecutively admitted and treated at the Department of Gynaecological Oncology at the University Hospital Groningen, follow-up data were available. Of these 367 patients 106 patients died of cancer.

In vitro translation.

pGEM1 plasmids containing the ORFs for E6 and E7 were kindly provided by dr P. Howley (described in 4 and 21). One μg of plasmid DNA was used for combined *in vitro* transcription and translation using the TnT™ Coupled Reticulocyte Lysate System (Promega, Leiden, The Netherlands), in the presence of 40-50 μCi of ^{35}S-methionine and 40 units of Ribonuclease inhibitor (RNA guard, Pharmacia, Woerden, The Netherlands). After incubation for 2 hours at 30°C, the lysate was run over a Sephadex G25 column (Pharmacia), fractions collected, and the size of the labelled protein confirmed by SDS polyacrylamide gel electrophoresis.

Detection of antibodies by RIPA.

The fraction containing the labelled protein was diluted to approximately 30,000 cpm/100 μl in RIPA buffer (10 mM Tris-HCl pH 8.0, 140 mM NaCl, 2.5 mg/100 ml NaN_3, 0.1% Nonidet P-40). Two μl of serum and 100 μl of labelled protein were mixed and incubated at 4°C for 16 hr. A 0.45 μm Multiscreen microtiterplate (mtp, Millipore, Molsheim, France) was prewetted with RIPA buffer and loaded with protein A sepharose (PAS, Sigma, St.

Louis, MO, USA, 0.15 g/plate) in RIPA buffer. Serum and lysate were transferred to the Multiscreen mtp and incubated at 4°C for 2 hr, whilst shaking. After 7 washes with RIPA buffer, the PAS was transferred to a fresh Multiscreen mtp, and washed 3 more times. The filters were punched out, transferred to scintillation vials containing 1.5 ml of InstaGel Plus (Packard, Groningen, The Netherlands), and cpm were read in a scintillation counter (Packard Tri-carb 460 CD). Each serum was tested in triplicate. In each plate positive and negative controls were included, as well as wells without serum (background control).

Statistical methods.

Odds ratios were calculated to compare the antibody prevalence between cervical cancer patients and controls. The association between anti E6 and/or E7 positivity in pretreatment sera from cervical carcinoma patients and tumour related variables were investigated using bivariate techniques (Pearson Chi-square test) and multivariate logistic regression analysis. The prognostic value of anti E6 and/or E7 antibodies and other possible risk factors was determined in Cox' regression model. The data used for analysis of the cervical carcinoma patient group included stage of disease (FIGO stage), lesion size, tumour grade, lymph node status, vascular invasion, depth of infiltration and the presence of antibodies against E6 and/or E7 protein.

Results

Prevalence of antibodies to HPV-16 E6 and E7 in patient groups and controls.

Seroreactivity to the various combinations of antigens in both patient groups and controls are shown in Table 1 and Figure 1. Cutoff values were calculated on the basis of results for the controls (mean + 3*SD) and were 188 cpm for E6 and 116 cpm for E7. High

Table 1. Prevalence of antibodies to HPV 16 E6 and E7 in patients with cervical carcinoma and controls by RIPA.

Antigen	CC Patients			Controls			CIN Patients			OR (95% ci)[1]
	N	pos	%	N	pos	%	N	pos	%	
E6 RIPA	383	102	26.6	195	8	4.1	111	3	2.7	8.5 (4.0-17.8)
E7 RIPA	388	121	31.2	195	9	4.6	111	3	2.7	9.4 (4.6-18.9)
E6 or E7	378	131	34.7	195	13	6.7	111	3	2.7	7.4 (4.1-13.6)
E6 and E7	378	92	24.3	195	4	2.1	111	3	2.7	15.4 (5.6-42.5)

[1] Odds ratios have been calculated for cervical carcinoma patients vs controls.
OR = odds ratio, ci = confidence interval.

values for RIPA E6 (mean + 5*SD) were exclusively found in cervical cancer patients, whereas one control had a high antibody level in RIPA E7. Antibody prevalences to E6, E7, E6 and/or E7 and E6 and E7 were significantly higher in cervical cancer patients as compared to both controls and CIN patients ($p < 0.001$). Odds ratios, calculated for cervical carcinoma patients versus controls, ranged from 7.4 to 15.4. There was a strong tendency for patients to have antibodies to both E6 and E7 simultaneously (Spearman's $r = 0.71$, $p < 0.001$, Figure 2).

HPV type specificity of anti-E6 and E7 antibodies.

From 137 of the 392 patients the HPV type in the tumour could be determined by PCR on paraffin-embedded tissue. We analyzed whether the presence of antibodies against E6 and/or E7 was related to the HPV type in the primary lesion. We compared the findings to those with antibodies detected by E7 synthetic peptide ELISA (E7 ELISA, Table 2). The seroprevalence of antibodies to E6 and/or E7 as detected by RIPA was significantly higher in the HPV-16 positive patients than in the non-HPV group (E6 RIPA, $p < 0.025$; E7 RIPA, $p < 0.05$). Furthermore, in this group of HPV-16 positive patients, antibody positivity was higher by E6 and/or E7 RIPA than by E7 ELISA. Finally, RIPA antibody positivity was found significantly more frequently in HPV-16 positive patients than in patients positive for other HPV types (E6 RIPA, $p < 0.01$; E7 RIPA, $p < 0.025$).

Table 2. Prevalence of antibodies to HPV 16 E6 and E7 in patients with a known HPV status.

Antigen	HPV negative			HPV type 16			HPV other types[1]		
	N	pos	%	N	pos	%	N	pos	%
E7 ELISA	19	1	5.3	85	22	25.9	33	7	21.2
E6 RIPA	19	1	5.3	85	33	38.8	31	4	12.9
E7 RIPA	19	2	10.6	85	33	38.8	33	5	15.2
E6 or E7	19	2	10.6	85	37	43.5	31	7	22.6
E6 and E7	19	1	5.3	85	29	34.1	31	2	6.5

[1] = other types include HPV types 6, 18, 31, 33, 34, 45 and 52.

Analysis of the relation between E6 and E7 antibodies in pretreatment sera from HPV-16 positive patients with cervical carcinoma and clinico-pathological parameters.

Based on the higher prevalence of E6 and/or E7 antibodies, the analysis was limited to 85 HPV-16 positive patients. No relation was found with differentiation grade, vascular invasion, and depth of invasion in any of the tests. Table 3 shows the prevalence of anti- E6 and/or E7 antibodies as determined with RIPA and ELISA in relation with tumour volume, lymph node metastasis and prognosis. Of the different serological assays only positivity for antibodies against E7 as detected by ELISA showed a significant relation to tumour volume ($p = 0.003$). Neither E6 or E7 RIPA positivity nor positivity for both E6 and E7 by RIPA showed a significant relation with tumour size. A significant relation with lymph node metastasis was obtained for patients positive for antibodies against both E6 and E7 as detected by RIPA, and for patients positive in E7 ELISA. All serological assays showed a relation close to significance (p-values 0.05 to 0.07) with the clinical outcome of patients.

Logistic regression analysis was performed to predict the presence of lymph node metastasis. Pretreatment variables included FIGO stage, lesion size, differentiation grade, vascular invasion, invasion depth, as well as E6 and E7 RIPA and E7 ELISA. It was shown that four variables contributed significantly to the prediction of lymph node metastasis; FIGO stage, lesion size, vascular invasion and E7 RIPA ($X^2=23.03$; df=6; p=0.0008; sensitivity 62.5%; specificity 91.11%).

Similarly, logistic regression analysis for the prognosis of the patient, based on the same pretreatment variables was performed. This showed that three variables contributed significantly to the prediction of patient outcome; FIGO stage, differentiation grade and E6 RIPA ($X^2=16.64$; df=6; p=0.0107; sensitivity 27.78%; specificity 94.92%). The other parameters did not have any additional value for the estimation of patient survival.

Table 3. Correlation of the presence antibodies to HPV 16 E6 and E7 in patients positive for HPV 16 in their primary tumour with clinico-pathological parameters.

	E7 ELISA		RIPA E6		RIPA E7		RIPA E6/E7		RIPAE6+E7	
	+/N	p-value	+/N	p-value	+/N	p-value	+/N	p-value	+/N	p-value
Tumourvolume										
< 4 cm	8/50		18/50		17/50		21/50		14/50	
≥ 4 cm	13/27	0.003	13/27	NS[1]	14/27	NS	14/27	NS	13/27	0.077
Lymph nodes										
negative	9/48		15/48		15/48		18/48		12/48	
positive	11/27	0.039	14/27	0.079	14/27	0.079	15/27	NS	13/27	0.041
Patient outcome										
NED[2]	14/66		22/66		22/66		25/66		19/66	
DOD[3]	8/19	0.07	11/19	0.053	11/19	0.053	12/19	0.05	10/19	0.053

[1] NS = not significant, values below 0.1 are given in full, [2] NED = no evidence of disease, [3] DOD = dead of disease.

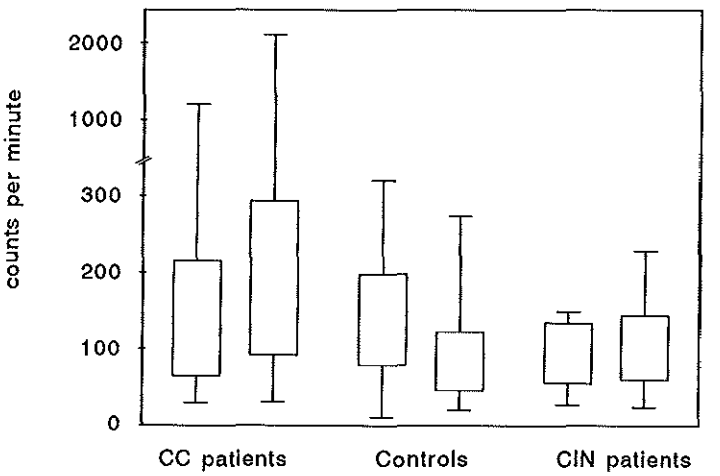

Figure 1: Distribution of cpm values of sera as determined by E6 and E7 RIPA.
X-axis studied groups
Y-axis antibody level in cpm
The length of the box corresponds to the interquartile range, with upper boundary representing the 75th, and the lower boundary the 25th percentile. The vertical line links the minimum and maximum values.

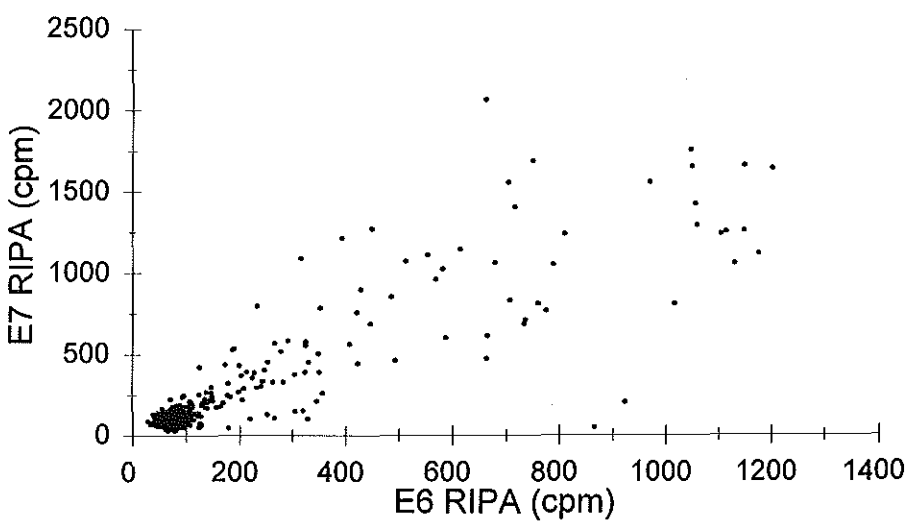

Figure 2: Correlation between E6 and E7 antibody levels as determined by RIPA.
X-axis anti-E7 antibody level in cpm
Y-axis anti-E6 antibody level in cpm
Spearman's r = 0.71, $p << 0.001$.

Discussion

Early HPV serology has shown that both sensitivity and specificity of synthetic peptide ELISA and fusion protein immunoblotting are too low to be of clinical significance. Therefore, the interest has shifted to the use of native proteins in various assays. Although a baculovirus expression system has been developed for both E6 and E7[15,16] most studies report the use of *in vitro* transcription/translation of E6 and E7[3,11,18-20]. Radio immunoprecipitation of the proteins generated by *in vitro* translation has shown a remarkable increase in both sensitivity and specificity. In the present study, investigating 392 patients with squamous cell carcinoma of the uterine cervix in E6 and E7 RIPA, we found both an increase in sensitivity when compared to E7 synthetic peptide ELISA (E7 RIPA 31.2%, E7 ELISA 17.7%[1]), and an increase of the specificity (E7 RIPA 95.4%, E7 ELISA 89%[1]). Furthermore, a slightly higher cut-off (mean + 5*SD) increased the specificity to nearly 100%. For E7 RIPA this is in accordance with other studies (sensitivity 19% - 43%, specificity 95% - 96%[3,18,19]). For E6 RIPA a sensitivity of 26.6%, and a specificity of 95.9% was found. A slightly higher sensitivity has been reported (37% - 56%[3,18,19]). We found that most patients have antibodies to E6 and E7 simultaneously, in contrast with the results of other investigators[18,19]. This discrepancy is striking, since it has been shown that E6 and E7 are co-expressed in cervical cancer cells and cancer-derived cell lines[14,17,22], suggesting simultaneous exposure to the immune system.

Antibody prevalence is related to the specific HPV type in the primary tumour. Due to the source of material, formaldehyde-fixed paraffin-embedded tissue, it is possible that patients negative in PCR do harbour low amounts of HPV DNA. Recently, the antagonistic effect of formaldehyde fixation due to DNA modification has been described[8]. In fact, one patient negative in HPV PCR was strongly positive in all three serological tests, suggesting this patient had an HPV-16 infection.

Because of the higher prevalence of antibodies in the E6 and E7 RIPA, analysis of the prevalence of antibodies against E6 and E7 to clinico-pathological parameters was limited to 85 HPV-16 positive patients. This inevitably leads to a reduction in statistical power. Nevertheless, in agreement with our previous finding[1], a significant relation between E7 ELISA and tumour volume ($p = 0.003$), as well as lymph node involvement ($p = 0.039$) was found. No relation was found between RIPA and the clinicopathological parameters. However, in nearly all comparisons the probability under the null-hypothesis was 0.05-0.08. In our opinion this finding indicates a relationship between antibodies against E6

and/or E7 and clinico-pathological parameters. Logistic regression analysis, including all three serological assays, showed the strongest relation between anti-E7 antibody positivity and lymph node involvement, and anti-E6 antibody positivity and survival. Analysis of all three serological assays separately, however, showed that there were only marginal differences between the predictive values of E6 and E7 positivity for both lymph node involvement and survival.

Since both E6 and E7 proteins are consistently and simultaneously expressed in cancer cells[14,17,22], a similar course of antibody expression for E6 and E7 can be expected in cervical cancer. To obtain a humoral immune response, E6 and E7 protein or fragments thereof, should be recognized by B-cells. For this to occur, E6 and E7 protein must be released from the HPV infected cells. This can be achieved either by necrosis of tumour tissue, or by a cellular immune attack on HPV infected cells. Our results suggest that the chance that E6 and E7 are released from HPV infected tumour cells increases with tumour load. The observed correlation between anti-E6 and anti-E7 positivity with lymph node involvement suggests that extension of the tumour beyond a certain barrier increases the chance of antibody production. Especially the cancer metastasis to the lymph nodes, as immunocompetent centres, may enhance a humoral immune response.

In conclusion, the results of this study underline our previous finding that antibody levels against the transforming proteins E7 and also E6 are related to tumour load and lymph node involvement. Further research is warranted to elucidate the immune response to HPV in cervical cancer as this may be of key importance in the immune response to the tumour.

Acknowldgements

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THE HUMORAL IMMUNE RESPONSE AGAINST THE TRANSFORMING PROTEINS E6 AND E7 IN HPV-16 POSITIVE CERVICAL CARCINOMA PATIENTS DURING TREATMENT AND FOLLOW-UP

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Submitted for publication

ABSTRACT.

Objective: To investigate the humoral immune response to the transforming proteins E6 and E7 of human papillomavirus (HPV) type 16 before and after treatment and during follow-up.

Methods: Consecutive serum samples from 36 patients, whose tumours were found to contain HPV 16 DNA by use of PCR, were tested using in vitro translated proteins E6 and E7 in a radioimmuno precipitation assay, and in a E7 synthetic peptide ELISA. Antibody levels were related to the clinical status of the patients.

Results: Seronegative patients remained seronegative throughout treatment and follow-up. Seropositive patients showed either a decrease in antibody level, or stable antibody levels during treatment. In contrast to patients without evidence of disease at the end of the study, the majority of patients with recurrent disease showed increasing antibody levels during follow-up.

Conclusion: In patients who are seropositive before treatment antibody levels against E6 and E7 of HPV type 16 are closely linked to treatment response. Recurrence of disease is associated with an increase in antibody level.

INTRODUCTION.

The association of human papillomaviruses (HPV) with cervical carcinoma has been established beyond doubt[1]. In over 90% of all carcinomas of the uterine cervix HPV DNA can be detected by the use of the polymerase chain reaction (PCR) on fresh tissue[2-5]. Although it has been postulated in a few reports that non-detection of HPV DNA[6], or detection of HPV type 18[7,8] has prognostic significance, other authors have suggested that the presence of HPV DNA does not have any prognostic significance[9,10]. On the other hand, the humoral immune response to one or more of the HPV proteins might provide serological markers that are associated with the outcome of disease.

HPV type 16, which is most frequently found in squamous cell cervical carcinomas, would appear the most suitable target for antibody detection, and indeed a number of authors have reported a significantly higher antibody prevalence to the HPV-16 transforming proteins E6[11-14] and E7[11-13,15-18] in cervical carcinoma patients than in controls. However, limited information is available concerning the serological follow-up of patients during treatment and follow-up. Lehtinen *et al.*[19] used a synthetic peptide for HPV 16 E2 to monitor 27 patients after treatment. Chee *et al.*[13] investigated antibody levels to native E6 and E7 in 14 patients pending treatment and during follow-up. Lenner *et al.*[20] studied IgA and IgG antibody levels in 66 patients to a variety of antigens, including HPV 16 E2, E7 and L1, and HPV 18 E2. Recently, we have observed that serum antibody levels to a synthetic peptide of HPV 16 E7 decreased during therapy[21]. In patients who clinically showed a complete remission, antibody levels fell to normal or remained stable during follow-up. However, in 4 out of 5 patients who were found to have a recurrence during follow-up an increase of antibody levels to E7 was observed. To extend the results obtained for the E7 synthetic peptide in the present study we have investigated the antibody levels during treatment and follow-up to both native E6 and E7 transforming proteins of HPV 16 in radio immuno precipitation assay (RIPA) and compared the results to the E7 synthetic peptide ELISA for 36 patients known to be positive for HPV 16 in their primary tumour. The results show that the antibody levels against the transforming proteins E6 and E7 of HPV 16 correlate with the clinical disease status in patients with positive pretreatment sera.

METHODS.

Pretreatment sera as well as sera drawn during treatment and follow-up from 36 women with squamous cell cervical carcinoma were obtained from the Serum Bank of the Department of Obstetrics and Gynaecology, University Hospital Groningen. Patients comprised a sample of women with HPV 16 DNA in the primary tumour as described elsewhere[22], from whom follow-up sera were available. Criteria for the judgment of the clinical course of the disease have been described previously[23]. Briefly, "complete remission" was defined as the absence of all tumour lesions 3 months after completion of treatment. "Residual disease" existed when tumour lesions were still known to be present 3 months after treatment. "No evidence of disease" (NED) was used for patients with complete remission after completion of treatment, who did not show signs of disease during follow-up. "Recurrent disease" was used to indicate the reappearance of disease in patients who experienced complete remission.

pGEM1 plasmids containing the ORFs for E6 and E7 were kindly provided by dr P. Howley, (described in references 24 and 25). One μg of plasmid DNA was used for combined *in vitro* transcription and translation using the TnT™ Coupled Reticulocyte Lysate System (Promega, Leiden, The Netherlands), in the presence of 40-50 μCi of ^{35}S-methionine (Amersham, Little Chalfont, UK) and 40 units of Ribonuclease inhibitor (RNA Guard, Pharmacia, Woerden, The Netherlands). After incubation for 2 hours at 30°C, the lysate was run over a Sephadex G25 column (Pharmacia, bedvolume approximately 8 ml), fractions collected, and the size of the labelled protein confirmed by SDS-polyacrylamide gel electrophoresis.

The fraction containing the labelled protein was diluted to approximately 30,000 cpm/100 μl in RIPA buffer (10mM Tris-HCl pH 8.0, 140 mM NaCl, 2.5 mg/100 ml NaN_3, 0.1% Nonidet P-40). Two μl of serum and 100 μl of labelled protein were mixed and incubated at 4°C for 16 hr. A 0.45 μm Multiscreen microtiterplate (mtp, Millipore, Molsheim, France) was prewetted with RIPA buffer and loaded with 200 μl protein A Sepharose suspension (PAS, Sigma, St. Louis, MO, USA, 0.15 g/20 ml RIPA buffer). Serum and lysate were transferred to the Multiscreen mtp and incubated at 4°C for 2 hr, whilst shaking. After 7 washes with RIPA buffer, the PAS was transferred to a fresh Multiscreen mtp, and washed 3 more times. The filters were punched out, transferred to scintillation vials containing 1.5 ml of InstaGel Plus (Packard, Groningen, The Netherlands), and cpm were read in a scintillation counter (Packard Tri-carb 460 CD).

Each serum was tested in triplicate. In each plate positive and negative controls were included, as well as wells without serum (background control). The mean value plus three times the standard deviation obtained from 195 healthy controls was taken as a threshold value for positivity in both E6 and E7 RIPA (values ≥ 150 cpm were considered positive).

Synthetic peptide ELISA was performed as described before[18,21]. Briefly, synthetic peptide HPV 16 E7\6-35 (100 µl per well at 5 µg/ml in PBS, pH 7.2) was coated onto 96 well Maxisorp plates (Nunc, Roskilde, Denmark) for 1 hour at 37°C. Control wells were treated in a similar way, with the omission of synthetic peptide from the buffer. The plates were blocked with 1 % non-fat milk in PBST (PBS containing 0.05 % Tween 20) for 1 hr at 37°C. Sera were incubated in a 1:100 dilution in PBST, 1 % non-fat milk for 1 hr at 37°C. Goat anti Human IgG peroxidase (1:6,700 in PBST, 1 % non-fat milk, Diagnostic Centre SSDZ) was used as a conjugate. After 1 hr at 37°C the plates were washed 6 times (PBST) followed by the addition of substrate (3,3',5,5'-Tetramethyl-benzidine, Aldrich Chemie, Bornem, Belgium). After 20 min the reaction was stopped with 3N H_2SO_4 and colour development read with a Titertek Plate reader at 450 nm. Each sample was tested in three independent experiments. In each experiment all sera were tested in duplicate on peptide-coated wells and control wells and the mean reactivity of the control wells was subtracted from the mean reactivity of the coated wells. For intra-test comparison, both positive, negative and borderline positive sera were included in each plate. For inter-test comparison, optical densities were recalculated into ratios by division by the cut-off value. A ratio of ≥ 1 was considered positive.

For statistical analysis of differences in antibody levels at different stages in one patient (pretreatment, posttreatment and follow-up) the T-test for paired samples was used.

RESULTS.

Consecutive serum levels of a total number of 36 patients with HPV 16 in their primary tumour were studied before and during treatment and during follow-up, using two methods; an E7 synthetic peptide ELISA and immunoprecipitation of radio-labelled HPV 16 E6 and E7 proteins.

Seven patients had negative serum antibody levels before treatment in all three tests, and remained negative throughout treatment and follow-up. Five of these seven patients showed no evidence of disease at the closing date of the study, whereas two patients experienced a recurrence of the tumour during follow-up.

Figure 1.

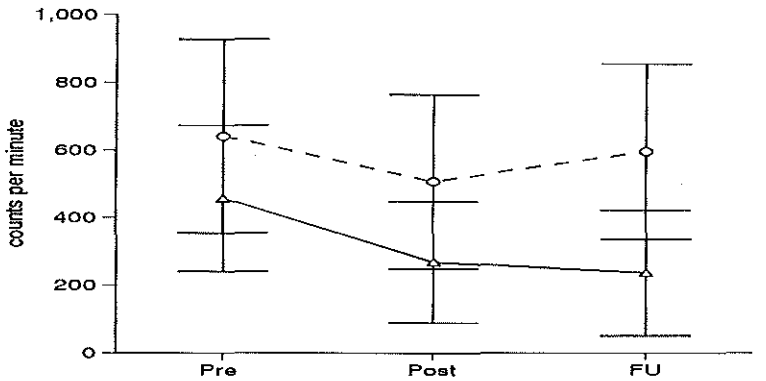


Figure 2.

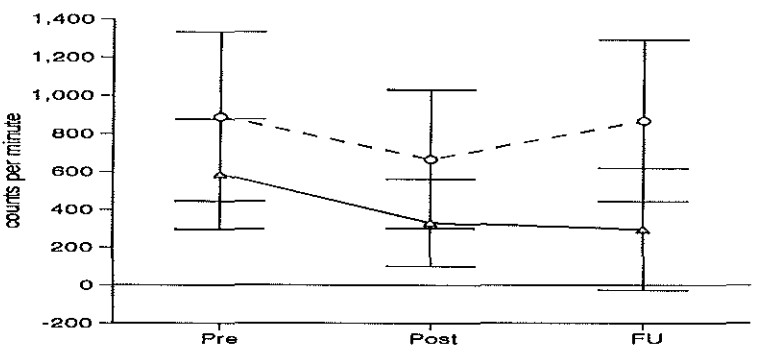


Figure 3.

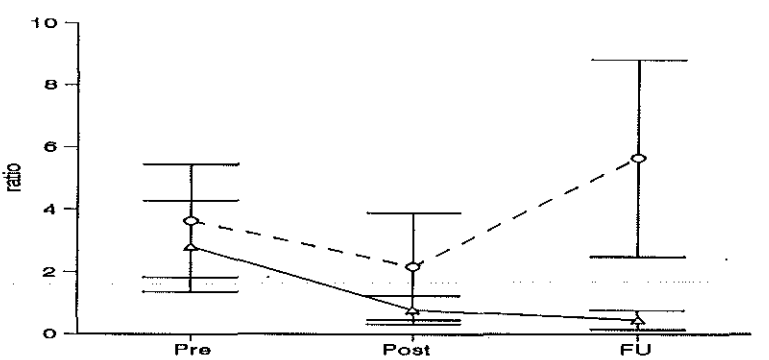
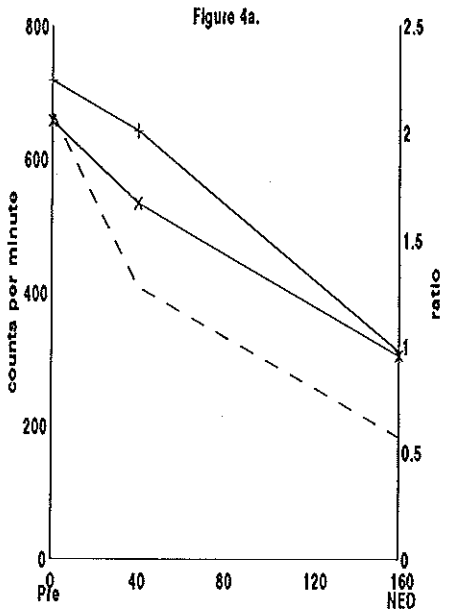


Figure 1: Mean antibody levels (± SD) as determined by E6 RIPA during and after treatment and during follow-up for all pretreatment seropositive patients.
-Δ-Δ- patients without evidence of disease at the end of the study.
-o-o- patients with a recurrence during follow-up.
Posttreatment sera were obtained between 2 and 9 months after therapy. For patients with NED the decrease in antibody level between pre (Pre) - and posttreatment (Post) sera was highly significant ($p < 0.001$). For patients with recurrent disease the decrease was also significant ($p < 0.05$). The increase in antibody level between posttreatment and follow-up serum (FU) in patients with recurrent disease was not significant.

Figure 2: Mean antibody levels (± SD) as determined by E7 RIPA during and after treatment and during follow-up for all pretreatment seropositive patients.
-Δ-Δ- patients without evidence of disease at the end of the study.
-o-o- patients with a recurrence during follow-up.
Posttreatment sera were obtained between 2 and 9 months after therapy. For patients with NED the decrease in antibody level between pre (Pre) - and posttreatment (Post) sera was highly significant ($p < 0.001$). For patients with recurrent disease the decrease was also significant ($p < 0.01$). The increase in antibody level between posttreatment and follow-up serum (FU) in patients with recurrent disease was not significant.

Figure 3: Mean antibody levels (± SD) as determined by E7 ELISA during and after treatment and during follow-up for all pretreatment seropositive patients.
-Δ-Δ- patients without evidence of disease at the end of the study.
-o-o- patients with a recurrence during follow-up.
Posttreatment sera were obtained between 2 and 9 months after therapy. For patients with NED and for patients with recurrent disease the decrease in antibody level between pre (Pre) - and posttreatment (Post) sera was highly significant ($p < 0.001$). The increase in antibody level between posttreatment and follow-up serum (FU) in patients with recurrent disease was significant ($p < 0.05$).



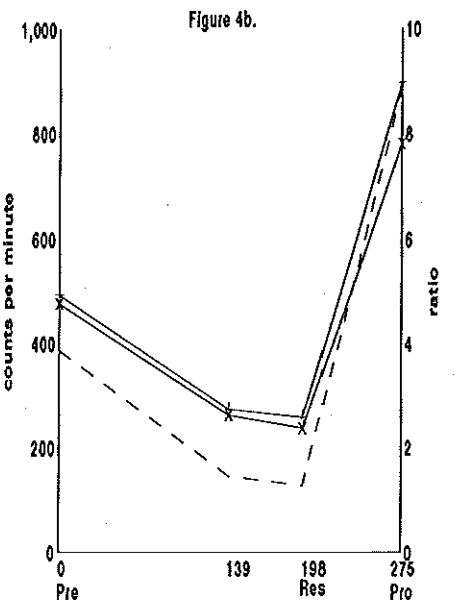


Figure 4a: Antibody levels as determined by E6 and E7 RIPA and by E7 ELISA during and after treatment and during follow-up in a patient (no. 41) with no evidence of disease (NED). The numbers on the x-axis indicate the number of days since the pretreatment serum (PRE) was taken.

RIPA E6	—x—	(cpm, left Y-axis)
RIPA E7	—+—	(cpm, left Y-axis)
E7 ELISA	- - - - -	(ratio, right Y-axis)

Figure 4b: Antibody levels as determined by E6 and E7 RIPA and by E7 ELISA during and after treatment and during follow-up in a patient (no. 38) with residual disease (RES) after treatment, followed by progression of disease (PRO). The numbers on the x-axis indicate the number of days since the pretreatment serum (PRE) was taken.

RIPA E6	—x—	(cpm, left Y-axis)
RIPA E7	—+—	(cpm, left Y-axis)
E7 ELISA	- - - - -	(ratio, right Y-axis)

All of the remaining 29 patients had positive pretreatment sera by E7 RIPA, 28 had positive pretreatment sera by E6 RIPA and 18 had positive pretreatment sera by E7 synthetic peptide ELISA.

After treatment 26 of the 29 patients were in complete remission, whereas 3 patients still showed residual disease. Of the 26 patients in complete remission 6 experienced recurrent disease during follow-up. Five of these 6 patients died of tumour progression, one patient is without disease following treatment of a recurrence in the vaginal vault.

Independent of clinical outcome, antibody levels to native E6 and E7 and E7 synthetic peptide decreased in most patients during treatment.

The mean levels of antibodies against native E6, native E7 and E7 synthetic peptide before and after treatment, and during follow-up, are shown in Figures 1-3. For patients with no evidence of disease during follow-up the reduction in antibody level after treatment was highly significant in all three serological tests ($p < 0.001$). For patients who experienced a recurrence of disease during follow-up the decrease in antibody level was significant in E6 RIPA ($p < 0.05$), E7 RIPA ($p < 0.01$), and E7 ELISA ($p = 0.001$).

The mean antibody levels against native E6, native E7 and E7 synthetic peptide did not show an increase following treatment in patients with no evidence of disease at the end of the study (follow-up 1-1.5 years). For patients who experienced a recurrence of disease a non-significant rise in mean antibody level as detected by E6 or E7 RIPA was found. However, the increase in antibody level against E7 synthetic peptide was significant ($p < 0.05$). Looking at the individual patients, during follow-up after treatment only one of the 20 patients with no evidence of disease at the closing date of the study showed an increase in antibody level. In contrast, the majority of patients who experienced a recurrence showed increasing antibody levels against native E6, (4/6 = 67%), native E7 (3/6 = 50%), and E7 synthetic peptide (4/4 = 100%).

In patients positive for antibodies against both E6 and E7 a similar pattern of antibody levels during treatment and follow-up was observed for all three antigens. Two examples are shown in figure 4a+b.

DISCUSSION.

To investigate the immune response against HPV 16 E6 and E7 during and after treatment and during follow-up in patients with squamous cell carcinoma of the uterine cervix, we have studied serial serum samples from 36 patients positive for HPV 16 in their primary

lesion. Serum samples were studied in an HPV 16 E7 synthetic peptide ELISA and in RIPA, using native proteins of HPV 16 E6 and E7.

As in our previous study[21] and reported by Chee *et al.*[13] and Lenner *et al.*[20], none of the patients negative in pretreatment serum showed seroconversion upon therapy, indicating that the immune response to E6 and E7 proteins is not changed due to the treatment procedures.

The decrease in antibody levels found in patients during and after therapy confirms our previous findings that antibody levels to E7 are linked to tumour load[18,21]. In a number of studies a decrease in antibody levels following treatment has been reported. Lehtinen *et al.*[19] studied the IgG and IgA antibody response against a synthetic peptide of HPV 16 E2 during treatment and follow-up in 27 patients with cervical carcinoma. The authors observed a decrease in antibody level to HPV 16 E2 following treatment in most early stage (FIGO I-II) patients, whereas a transient decrease was found in patients with Stage III disease. Lenner *et al.*[20], using an ELISA for synthetic peptides derived from HPV 16 E2, E7 and L1, and HPV 18 E2, reported a significant difference in IgG antibody levels against all antigens tested between pre- and post-treatment sera. Chee *et al.*[13] examined the change in antibody titer to native E6 and E7 of HPV 16 in a small number of cervical carcinoma patients. In 4 patients, seropositive against HPV 16 E7 at diagnosis, antibody levels showed a decrease upon treatment. However, the levels of antibodies against HPV 16 E6 did not show any significant variation along with the clinical response in 5 of 7 patients studied. This finding is in contrast with the results of our study. The reason for this discrepancy is not clear. Since both E6 and E7 are consistently expressed in cervical carcinoma cells, a similar course of antibody response for both proteins upon treatment would be expected. We have recently reported[18] that the presence of antibodies against HPV 16 E7 in pretreatment sera from cervical carcinoma patients correlates with the size of the tumour. Follow-up studies described above[13,20,21] further underline the relation of antibody levels against HPV 16 E7 and tumour load.

During follow-up an increase in antibody level to HPV 16 E6 and E7 was found in most patients with recurrence or progression of disease. Only one out of 20 patients with no evidence of disease showed an increase in antibody level to E6 and E7 during follow-up. This patient had normal SCC-antigen and CA 125 levels, two well-known prognostic tumour markers in cervical carcinoma[23,26]. Reports in literature concerning the antibody response against HPV proteins during follow-up are scarce and restricted to only a few patients. Chee *et al.*[13] reported an increase in antibody levels to HPV 16 E7 during follow-

up in 1 patient with recurrent disease, whereas 3 patients without evidence of disease during follow-up did not show an increase. In our previous study 4 out of 5 patients with a recurrence during follow-up showed an increase in anti-E7 antibody level, compared to none of six patients without evidence of disease during follow-up[21].

In conclusion, our results and those of Chee *et al.* clearly show that in the individual patient seropositive before treatment, the antibody level against E6 and E7 is related to treatment response, and that recurrence of disease is associated with an increase in antibody level. Further research will be necessary to assess the clinical value of these findings.

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DETECTION OF HPV-16 DNA BY PCR IN HISTOLOGICALLY CANCER-FREE LYMPH NODES FROM CERVICAL CANCER PATIENTS

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The association between the presence of human papillomavirus (HPV) DNA and cervical carcinoma has been established beyond doubt[1]. Presence or absence of pelvic lymph node involvement is one of the strongest predictive factors for prognosis of the cervical cancer patient[2,3]. A number of studies has investigated the presence of HPV DNA in lymph nodes from patients with HPV DNA in their primary tumour[4-7] and found HPV DNA positivity ranging from 14.3% to 50% in histologically negative lymph nodes. However, the prognostic significance could not be established in these reports due to the lack of follow-up data.

In order to investigate the frequency of detectable HPV DNA in histologically cancer-free lymph nodes, we examined 50 patients with squamous cell cervical carcinoma treated at the Department of Obstetrics and Gynaecology, University Hospital Groningen. From these patients paraffin-embedded tissue of the left obturator lymph nodes was investigated; per patient one lymph node was examined. All patients were positive for HPV-type 16 in their primary tumour. All 50 obturator lymph nodes examined were histologically negative for tumour cells. However, 15 patients had histological evidence for metastasis to one or more other lymph nodes. DNA was extracted from 10 μm sections by deparaffination and digestion with proteinase K as described by Wright and Manos[8]. Adjacent sections were haematoxylin and eosin (HE) stained. As a control for DNA extraction ß-globin primers PC03/04 (ACACAACTGTGTTCACTAGC / CAACTTCATCCACGTTCACC) were used. For detection of HPV-16 DNA a primer pair (GGTCGGTGGACCGGTCGATG / GCAATGTAGGTGTATCTCCA, described in ref. 9), spanning a 96 basepair DNA sequence of the E6 open reading frame, was used. PCR amplification was performed according to the method of Saiki *et al.*[10]. The final 100 μl PCR reaction contained 10 μl sample, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X100, 0.01% gelatin, 200 μM dNTPs, 50 pmole of each primer and 0.25 units of SuperTaq (Sphaero Q, Cambridge UK). Standard PCR conditions are 1 min 95°C, 1 min 52°C and 2 min 72°C, for 40 cycles. After PCR 15 μl of all samples were run on a 2% agarose gel, gels were blotted (Hybond N^+, Amersham, Little Chalfont, UK) and hybridised with random labelled HPV-16 E6. Blots were washed twice for 15 min in 2*SSC, 0.1% SDS at 42°C.

All obturator lymph nodes were positive in the ß-globin PCR (100 bp DNA fragment). A total of 19 (38%) of the obturator lymph nodes were positive for HPV-16 DNA (table 1). HPV-16 DNA was detected in 7 of the 15 patients (46.6%) with known lymph node involvement elsewhere, compared to 12 of the 35 patients (34.3%) without histological

evidence for lymph node involvement. No difference was found between these groups of patients ($p = 0.61$; X^2 test with Yates' correction).

Table 1. HPV-16 DNA in the left obturator lymph node.

Cancer cells in other lymph nodes	HPV-16 DNA positive	negative
yes	7	8
no	12	23

In approximately one third (12/35) of the patients without histological evidence for lymph node involvement HPV-16 DNA could be detected in the lymph node examined. This is in accordance with data from the literature where HPV-DNA positivity in histologically negative lymph nodes ranging from 14.3% to 50% has been reported[4-7].

Burnett *et al.*[11] assign a prognostic value to the detection of HPV DNA in histologically negative lymph nodes. This assumption has been based on the finding that in 6 patients with recurrent disease 22 of 31 (71%) histologically negative lymph nodes were positive for HPV DNA, versus 15 of 43 (35%) histologically negative lymph nodes from 6 patients in complete remission. It is not clear, however, whether the number of HPV DNA positive nodes in the individual patient is related to the clinical outcome[11]. Nawa *et al.* reported recurrence of disease in 2 out of 5 HPV DNA lymph node positive patients without histological evidence of lymph node involvement. In the present study, however, only one of 12 HPV-16 DNA lymph node positive patients without histological evidence for lymph node involvement developed a recurrence. All other patients still show no evidence of disease (median time of follow-up = 47.5 months; range = 24-132 months). In the study by Burnett *et al.*[11] 5 of 6 patients with a recurrence were positive for HPV-18. This HPV type has been described as biologically more aggressive than HPV type 16[12-14]. Therefore, it cannot be ruled out that detection of HPV-18 DNA in histologically negative lymph nodes may be of prognostic value. The presence of HPV-DNA in histologically negative lymph nodes may be due to subclinical metastasis of tumour cells. Alternatively it may represent DNA from tumour cells destroyed by immune competent cells. Possibly, detection of HPV-specific m-RNA might give an answer to this question. Unfortunately, too little material was available to perform such an analysis.

In conclusion, no prognostic value has been found for the detection of HPV DNA in histologically cancer-free lymph nodes from patients with HPV-16 positive primary tumours.

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GENERAL DISCUSSION.

GENERAL DISCUSSION.

The studies presented in this thesis describe the humoral immune response against the transforming proteins E6 and E7 of human papillomavirus (HPV) type 16 in cervical carcinoma patients and investigate the possible use of the presence of antibodies to these proteins as diagnostic and/or prognostic markers.

As the work described in this thesis started, a number of studies[11,12,14,15,20,26] had already shown a significantly higher prevalence of antibodies against transforming proteins E6 and E7 of HPV-16 in cervical cancer patients in comparison with control groups and patients with CIN lesions. To compare the patient population in our study with those described by others, the prevalence of antibodies against the transforming protein E7 was investigated in a synthetic peptide ELISA in cervical cancer patients, age- and sex-matched controls, and patients with CIN lesions (chapter 2). The antibody prevalence was significantly higher in cervical carcinoma patients than in both controls and CIN patients, and was comparable to the observations by others[15,20]. However, the prevalence was too low to be of clinical relevance.

The lack of sensitivity of antibody detection by synthetic peptide ELISA was attributed to the absence of conformational epitopes in the relatively short synthetic peptide. This assumption has led to the development of assays incorporating native proteins. E6 and E7 proteins have been produced in the baculovirus expression system[28,29], and in *in vitro* transcription/translation assays[5,32]. In chapter 5 the sensitivity and specificity of detection of antibodies against HPV-16 E6 and E7 for the presence of cervical cancer was compared in different assay systems; the E7 synthetic peptide ELISA, with non-conformational epitopes, and radioimmuno precipitation of in vitro transcribed and translated E6 and E7 native proteins. It was shown that the use of native proteins in radioimmuno precipitation assays (RIPA) resulted in both a higher sensitivity and a higher specificity than the E7 synthetic peptide ELISA. Similar results have been observed by others[30].

In order to investigate the type-specificity of HPV-16 E6 and E7 antibody detection, the presence and type of HPV in the primary tumour of 162 patients was analyzed by PCR, using paraffin-embedded tissue. Several authors have reported difficulties in reproducing

PCR results with formaldehyde-fixed tissues[1,2,8,31]. The antagonistic effect of formaldehyde fixation due to DNA modification, causing the inhibition of PCR amplification of longer (>200 bp) amplimers, has been described by Karlsen *et al.*[13]. The nature of this material, therefore, necessitated the development of new type-specific primer sets for the detection of HPV-16, -18, -31 and -33 (chapter 4). These new primer sets resulted in short amplimers of about 100 base pairs, and enhanced the detection of HPV DNA considerably.

Antibodies to HPV-16 E6 and E7 were almost exclusively found in patients positive for HPV DNA in their primary tumour (chapter 5). Although the highest prevalence of antibodies against E6 and E7 was found in the HPV-16 DNA positive group, a substantial percentage of patients positive for other types of HPV had antibodies against HPV-16 E6 and/or E7. Detection of antibodies against HPV-16 E6 and E7, either by ELISA or RIPA, therefore, does not seem to be completely type-specific. Alternatively, the existence of double-infections cannot be ruled out altogether. Although the general belief is that outgrowth of the tumour is the result of clonal expansion of a single HPV-infected and subsequently genetically deranged cell, double-infections in cervical carcinoma patients are occasionally found. In our study of 137 patients, two patients had a double-infection (chapter 3). Little information from other studies is available on the frequency of double-infections in cancer patients.

Both ELISA and RIPA-positivity in cervical cancer patients were related to clinico-pathological parameters, such as tumour volume, infiltration depth, differentiation grade, vascular invasion and lymph node metastasis as well as clinical outcome (chapters 2 and 5). Antibody positivity in E7 synthetic peptide ELISA was significantly related to tumour volume, lymph node involvement and prognosis. Using RIPA for detection of antibodies against HPV-16 E6 and E7, in nearly all comparisons the probability under the null-hypothesis was 0.05-0.08. In our opinion this finding indicates a relationship between anti-E6 and/or anti-E7 positivity and tumour volume, lymph node metastasis and clinical outcome. This decreased relationship obtained for RIPA antibody positivity in comparison with ELISA positivity can be explained by the greater sensitivity of RIPA, leading to an increase in antibody positivity mainly in patients with small tumours or without lymph node involvement. Development of serological assays with enhanced sensitivity for the detection of anti-E6 and anti-E7 antibodies in cervical cancer patients, therefore, does not result in a stronger prognostic value. Although the use of a higher cutoff level results in stronger

association of the presence of anti-E6 and/or anti-E7 antibodies with clinicopathological parameters, this will lead to a reduced sensitivity, comparable to the sensitivity obtained with the E7 synthetic peptide ELISA.

Few data are available on the relation between antibodies and clinical parameters other than stage. Onda *et al.*[22] found that lymph node and distant metastases are more frequent in E7 antibody positive than in E7 antibody negative patients. Lenner *et al.*[18] were not able to find an association between pretreatment antibody levels and clinical stage or histopathological differentiation using a number of antigens including E7 synthetic peptides. Gaarenstroom *et al.*[7] showed that high levels of anti-E7 antibodies are associated with a shorter disease-free interval and poor survival. This association was lost after adjustment for stage, tumour volume and/or age. Our data indicate that the chance that antibodies against E6 and E7 are formed, increases with tumour load. To obtain a humoral immune response, E6 and E7 protein or fragments thereof, should be recognized by B-cells. For this to occur, E6 and E7 protein must be released from the HPV infected cells. This can be achieved either by necrosis of tumour tissue, or by a cellular immune attack on HPV infected cells. The observed relation between anti-E6 and anti-E7 positivity and lymph node involvement suggests that extension of the tumour increases the chance of antibody production. Especially cancer metastasis to lymph nodes, as immunocompetent centres, may enhance a humoral immune response. Furthermore, the observation that cervical cancer patients who have a humoral immune response to HPV-16 E6 and/or E7 have a worse prognosis than patients without a humoral immune response might be indicative of a difference in local cellular immune response. Tumour infiltrating lymphocytes may express distinct cytokine patterns. T-helper-1 cells produce a.o. IL-2 and IFN-gamma, whereas Th2 cells produce a.o. IL-4 and IL-10. Type-1 cytokines promote macrophage activation and an anti-HPV-16 CTL response, whereas type 2 cytokines favour the humoral immune response, with concomitant suppression of cell-mediated immune responses[16,19,24,27]. A Th1 response may therefore be able to eradicate the tumour or precancerous lesion by the CTL attack on HPV-infected cells, whereas a Th2 response may lead to progression of the tumour, as antibodies are insufficient to clear HPV-infected cells.

The antibody response against HPV-16 E6 and E7 was investigated in cervical cancer patients during and after treatment and during follow-up (chapters 3 and 6). Patients who were seronegative before treatment did not produce antibodies to E6 and/or E7 after

treatment, indicating that treatment does not alter the exposure of these two proteins to the immune system. In both RIPA and ELISA it was shown that after treatment antibody levels decreased in the majority of patients who were seropositive before treatment. The remaining patients had constant antibody levels. A number of studies[4,9,17,18] has shown a similar decrease in antibody level after treatment. However, apart from a single case-report[4], no data are available on antibody levels during follow-up after treatment. The results presented in this thesis show that during follow-up increasing antibody levels against HPV-16 E6 and E7 are associated with recurrence of disease. This strongly suggests a relation between anti-E6 and -E7 antibody levels and outgrowth of the tumour.

Finally, in view of the importance of lymph node involvement as a prognostic factor for survival, we investigated the presence of HPV-16 DNA in histologically negative lymph nodes from cervical cancer patients with HPV-16 DNA in their primary tumour (chapter 7), in order to assess the prognostic value of HPV DNA detection in cancer-free lymph nodes. Potentially, information on the presence of HPV DNA in resected lymph nodes may provide a means to identify patients who might benefit from adjuvant post-operative therapy. Lack of follow-up data precluded analysis of the prognostic significance in a number of studies[6,21,23,25]. Burnett *et al.*[3] assigned prognostic significance to the detection of HPV-18 DNA in histologically cancer-free lymph nodes. In contrast, neither in our study, nor in a recent study by Hording *et al.*[10], any predictive value was found for the detection of HPV DNA in cancer free lymph nodes.

In conclusion, in all serological assays, the antibody prevalence was significantly higher in cervical carcinoma patients than in both controls and CIN patients. Although RIPA for antibodies against HPV-16 E6 or E7 shows high specificity, sensitivity of anti-E6 and -E7 antibody detection in cervical cancer is too low (approximately 40%) to be useful as a diagnostic assay. Nevertheless, antibody positivity to both E6 and E7 is related to tumour characteristics, such as tumour volume and lymph node involvement as well as survival, suggesting a possible prognostic value. Therefore, antibodies against E6 and E7 do not seem to be protective. Analysis of antibody levels in patients after treatment and during follow-up has shown that in patients who are antibody positive before treatment, an increase in anti-E6 or anti-E7 antibody level during follow-up is related to the clinical outcome. This, and the relation between antibody positivity and a worse prognosis suggests that it might be beneficial to follow-up the antibody levels of patients who are antibody

positive before treatment.

The results of this study give some suggestions for further research. First of all, the follow-up of anti-E6 and anti-E7 antibodies in patients showed favourable results. To assess the prognostic value of follow-up of cervical cancer patients definitily, however, larger numbers of patients need to be studied. Secondly, the presence of antibodies against E6 and/or E7 was shown to be related to important clinicopathological parameters, such as tumour volume and lymph node metastasis. Detection of antibodies was limited to IgG in serum. The uterine cervix is guarded immunologically by the mucosal-associated lymphoid tissue, which forms a separate interconnected secretory system. Therefore, it would be most interesting to study the local production of antibodies, of the IgG as well as the IgA class. Sofar, little is known about this. Finally, the observation that women who have a humoral immune response to HPV-16 E6 and/or E7 have a worse prognosis than women without a humoral immune response might be indicative of a difference in local cellular immune response, i.e. a Th2 response in patients positive for anti-E6 and/or E7 antibodies and a Th1 response in patients negative for anti-E6 and/or E7 antibodies. The definitive proof for this hypothesis lies in the detection of the T helper cell profile in the actual cervical tissue. This hypothesis also urges research into the possibility to stimulate patients to switch from a predominant Th2 response to a predominant Th1 response in order to eradicate the tumour.

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SUMMARY

This thesis describes the humoral immune response against the transforming proteins E6 and E7 of human papillomavirus (HPV) type 16 in cervical carcinoma patients and investigates the possible use of the presence of antibodies to these proteins as diagnostic and/or prognostic markers.

In the first chapter the current knowledge on cervical cancer, human papillomaviruses and the papillomavirus immunology is reviewed. Cervical cancer is one of the most common cancers worldwide. Early detection and treatment of the disease lead to high 5-year survival rates for cervical cancer. The association of HPV, especially types 16, 18, 31 and 33, with cervical cancer suggests possible applications of the immune response to HPV proteins in diagnosis, treatment, and prevention. *In vitro* experiments have shown that the E6 and E7 proteins play an important role in carcinogenesis, which makes these proteins interesting antigens in serological tests. It has already been shown that antibodies against the E6 and E7 proteins occur significantly more often in cervical cancer patients than in controls.

In chapter 2, the relation between antibodies to the transforming protein E7 of HPV type 16 and clinico-pathological parameters in women with cervical squamous cell carcinoma was investigated, using an ELISA based on a synthetic peptide of the HPV type 16 E7 protein (amino acids 6 to 35). Cervical carcinoma patients had a significantly higher prevalence rate of antibodies to synthetic peptide E7\6-35 than women with cervical intra-epithelial neoplasia or controls. Bivariate analysis of the data on the presence of anti-E7\6-35 antibodies in the pretreatment sera from these patients and clinico-pathological parameters revealed a significant relation between the presence of anti-E7\6-35 antibodies and the size of the lesion, histological grade, and lymph node metastasis. In addition, the Cox' regression model revealed a significant relation between the presence of anti-E7\6-35 antibodies and a worse prognosis. Survival analysis showed that both for all FIGO stages, and stages IB and IIA alone, anti-E7\6-35 positive patients before treatment had a significantly shorter life expectancy.

Using the same synthetic peptide of HPV-16 E7 in ELISA, sera obtained from 31 cervical carcinoma patients were tested before and during treatment, and in follow-up, for the presence of anti-E7\6-35 antibodies (chapter 3). Sixteen patients with negative pretreatment

serum determination remained negative during treatment and follow-up. Of the 15 patients with positive pretreatment sera, 12 showed a decrease in anti-E7\6-35 antibody level during treatment. During follow-up an increase in anti-E7\6-35 antibody level was observed in 6 out of 7 patients with progressive or recurrent disease, whereas all patients who remained in complete remission showed stable or further decreasing antibody levels. The results suggest that in a subset of cervical cancer patients the anti-E7\6-35 antibody response against HPV-16 E7, at least partially, depends on the presence of viable tumor lesions. In this group of patients the anti-E7 profile reflects the course of disease.

In chapter 4 the efficacies of three general primer pairs, as well as type-specific primers for HPV type 16, 18, 31 and 33, were compared for the detection of HPV DNA in formaldehyde-fixed paraffin-embedded carcinomas. The use of the general primer pairs leads to an underestimate of the HPV prevalence. The efficacy of each general primer pair seemed to be inversely correlated to the length of the amplimer produced. Formaldehyde fixation limits the applicability of PCR due to DNA modification, which causes the inhibition of PCR amplification of longer (>200 bp) amplimers. Therefore, we developed new type-specific primer pairs (amplimer length approximately 100 bp), which led to detection of a higher HPV DNA prevalence.

In chapter 5 we investigated the clinical relevance of the enhanced sensitivity of antibody binding to *in vitro* translated HPV-16 E6 and E7 proteins in radio immunoprecipitation assays (RIPA) over synthetic peptide ELISA. Antibody prevalence to E6 and E7 in RIPA was significantly higher in cervical cancer patients as compared to both controls and CIN patients. Antibodies to E6 and/or E7 were largely restricted to patients with HPV DNA in their primary tumour. Analysis of the relation between the prevalence of antibodies to E6 and E7 and clinico-pathological parameters was limited to 85 HPV-16 positive patients. The strongest relation with clinico-pathological data, such as lesion size and lymph node involvement, as well as prognosis was found for E7 synthetic peptide ELISA, whereas E6 and E7 RIPA did not reach significance, indicating that development of serological assays with enhanced sensitivity for the detection of anti-E6 and anti-E7 antibodies in cervical cancer patients does not result in a higher prognostic value.

Consecutive serum samples from 36 patients, whose tumours were found to contain HPV 16 DNA by use of PCR, were tested using *in vitro* translated proteins E6 and E7 in RIPA,

and in a E7 synthetic peptide ELISA to investigate the humoral immune response to transforming proteins E6 and E7 of HPV type 16 before and after treatment and during follow-up (chapter 6). Seronegative patients remained seronegative throughout treatment and follow-up. Seropositive patients showed either a decrease in antibody level, or stable antibody levels during treatment. In contrast to patients without evidence of disease at the end of the study, the majority of patients with recurrent disease showed increasing antibody levels during follow-up, indicating that in patients who are seropositive before treatment antibody levels against E6 and E7 of HPV type 16 are closely linked to treatment response.

In order to investigate the frequency of detectable HPV DNA in histologically cancer-free lymph nodes, paraffin-embedded tissue of the left obturator lymph node from 50 patients with HPV-16 DNA positive squamous cell cervical carcinoma was examined using the newly developed HPV-16 specific primer pair (chapter 7). HPV-16 DNA was detected in 7 of the 15 patients with known metastasis to (a) lymph node(s) other than the obturator lymph node, compared to 12 of the 35 patients without histological evidence for lymph node involvement. As only one of 12 HPV-16 DNA lymph node positive patients without histological evidence for lymph node involvement developed a recurrence, no prognostic value was found for the detection of HPV-16 DNA in histologically cancer-free lymph nodes from patients with HPV-16 positive primary tumours.

In conclusion, in all serological assays, the HPV-16 E6 and/or E7 antibody prevalence was significantly higher in cervical carcinoma patients than in both controls and CIN patients. However, the results of antibody detection obtained by using either ELISA or RIPA indicate that the prevalence of antibodies in cervical cancer patients is too low, to be usefull as a diagnostic assay. Nevertheless, antibodies to both E6 and E7 are related to tumour characteristics, such as tumour volume and lymph node involvement, as well as survival, suggesting a prognostic value. Analysis of antibody levels in patients after treatment and during follow-up has shown that in patients who are antibody positive before treatment, an increase in anti-E6 or anti-E7 antibody level during follow-up is related to clinical outcome. This, and the relation between antibody positivity before treatment and a worse prognosis suggest that it might be beneficial to follow-up the antibody levels of patients who are antibody positive before treatment. Due to the relatively small number of patients investigated, further research to assess the value of follow-up of anti-E6 and anti-E7 antibodies in cervical cancer patients is warranted.

SAMENVATTING

Dit proefschrift beschrijft de humorale immuunrespons tegen de transformerende eiwitten E6 en E7 van humaan papillomavirus (HPV) type 16 in cervixcarcinoompatiënten (baarmoederhalskanker) en toetst het mogelijke gebruik van de aanwezigheid van antistoffen tegen deze eiwitten als diagnostische en/of prognostische markers.

In het eerste hoofdstuk wordt de huidige kennis aangaande baarmoederhalskanker, humane papillomavirussen en papillomavirus immunologie weergegeven. Wereldwijd gezien is baarmoederhalskanker één van de meest voorkomende kankervormen. Vroege detectie en behandeling van de ziekte leiden tot een hoge 5-jaars overleving. De associatie van HPV, met name de types 16, 18, 31 en 33, met cervixcarcinoom suggereert een mogelijke toepasbaarheid van de immuunrespons tegen HPV eiwitten in diagnose, behandeling en preventie. *In vitro* experimenten hebben aangetoond dat de E6 en E7 eiwitten een belangrijke rol spelen in de carcinogenese, hetgeen deze eiwitten tot interessante antigenen in een serologische test maakt. In de literatuur is beschreven dat antistoffen tegen de transformerende eiwitten E6 en E7 van HPV-16 significant vaker worden aangetroffen bij vrouwen met baarmoederhalskanker dan bij vrouwen uit controlegroepen.

In hoofdstuk 2 is de relatie onderzocht tussen antistoffen tegen het transformerende eiwit E7 van HPV-16, bepaald met behulp van ELISA gebaseerd op een synthetisch peptide van HPV-16 E7 (aminozuren 6-35), en clinico-pathologische parameters in cervixcarcinoom patiënten. De antistof prevalentie was significant hoger in de groep cervixcarcinoom patiënten dan in zowel de controle groep, als de groep CIN patiënten (een voorstadium van het cervixcarcinoom). Bivariate analyse toonde een significante relatie aan tussen de aanwezigheid van antistoffen en de grootte van de laesie, de histologische graad en lymfkliermetastase. Cox' regressie analyse toonde daarnaast een significante relatie aan tussen de aanwezigheid van antistoffen en een slechte prognose. Analyse van de overlevingscurves gaf aan dat zowel voor alle FIGO stadia, als voor stadia IB en IIA, patiënten met antistoffen tegen E7\6-36 vóór behandeling een significant kortere levensverwachting hadden.

Met behulp van hetzelfde HPV-16 E7 peptide in ELISA werd in sera van 31

cervixcarcinoom patiënten (verkregen vóór en gedurende behandeling en gedurende follow-up) de aanwezigheid van antistoffen tegen dit peptide bepaald (hoofdstuk 3). Zestien patiënten die geen antistoffen tegen HPV-16 E7 hadden vóór behandeling bleven negatief gedurende de behandeling en follow-up. Van de 15 seropositieve patiënten vóór behandeling, vertoonden 12 een daling in antistofniveau gedurende behandeling. Tijdens follow-up werd een stijging in antistof niveau waargenomen in 6 van de 7 patiënten met progressieve ziekte of een tumorrecidief, terwijl in patiënten zonder aantoonbare ziekte een verdere daling of gelijkblijvende antistofniveaus werden waargenomen. Deze resultaten suggereren dat in een deel van de cervixcarcinoom patiënten de immuunrespons tegen het HPV-16 E7 synthetisch peptide afhankelijk is van de aanwezigheid van actieve tumor laesies. In deze groep patiënten geeft het antistofprofiel tegen HPV-16 E7 het verloop van de ziekte weer.

In hoofdstuk 4 is de efficiëntie van HPV DNA detectie met behulp van PCR onderzocht met een drietal 'general primer sets' en type-specifieke 'primer sets' voor HPV typen 16, 18, 31 en 33 in formaldehyde-gefixeerd, paraffine-ingebed tumorweefsel. Het gebruik van de 'general primer sets' leidt tot een onderschatting van de HPV DNA prevalentie. De efficiëntie van de 'general primer sets' was omgekeerd evenredig met de lengte van het in de PCR geproduceerde amplimeer. Formaldehyde fixatie heeft een nadelig effect op PCR. Dit is te wijten aan DNA modificatie, hetgeen een remming van PCR amplificatie van langere amplimeren (groter dan 200 baseparen) tot gevolg heeft. Daarom hebben wij nieuwe HPV type-specifieke 'primer sets' ontwikkeld met een amplimeer lengte van ongeveer 100 baseparen. Het gebruik van deze 'primer sets' resulteerde in de detectie van een hogere HPV DNA prevalentie.

Om de klinische relevantie van de grotere gevoeligheid van antistofdetectie met behulp van radio immuno precipitatie assays (RIPA) ten opzichte van de synthetisch peptide-ELISA te bepalen, werd de RIPA uitgevoerd met HPV-16 E6 en E7 eiwitten (hoofdstuk 5). De antistof-prevalentie tegen E6 en E7 bleek in RIPA significant hoger in cervixcarcinoom patiënten dan in controles en CIN patiënten. Antistoffen tegen E6 en/of E7 werden vrijwel alleen aangetoond bij patiënten met HPV DNA in de tumor. Analyse van de relatie tussen antistof-prevalentie tegen E6 en E7 en clinico-pathologische parameters werd uitgevoerd voor 85 patiënten met HPV-16 in de primaire tumor. De sterkste relatie met clinico-pathologische parameters, zoals tumorgrootte en lymfkliermetastase, alsmede prognose

werd gevonden voor de E7 synthetisch peptide ELISA, terwijl de E6 en E7 RIPA niet significant waren gerelateerd met voornoemde parameters. Het ontwikkelen van serologische testen met een verhoogde gevoeligheid voor antistoffen tegen E6 en E7 gaat derhalve niet gepaard met een sterkere prognostische waarde.

Om de humorale immuunrespons tegen de transformerende eiwitten E6 en E7 vóór en na behandeling en gedurende follow-up te onderzoeken, werden opeenvolgende sera van 36 patiënten, in wier tumor HPV-16 DNA was aangetoond met behulp van PCR, getest in RIPA met E6 en E7 eiwitten, en de E7 synthetisch peptide-ELISA (hoofdstuk 6). Patiënten bij wie vóór behandeling geen antistoffen konden worden aangetoond, bleven seronegatief na behandeling. In de meerderheid van de patiënten die seropositief waren vóór behandeling, vond een afname plaats van de hoeveelheid antistoffen na behandeling. Alle overige patiënten hadden een gelijkblijvend antistofniveau. In tegenstelling tot patiënten zonder aantoonbare laesies, vertoonde de meerderheid van patiënten met een tumorrecidief een toename in antistofniveau gedurende follow-up. Dit wijst op een relatie tussen anti-E6 en anti-E7 antistofniveau's en klinisch beloop bij patiënten die seropositief zijn vóór behandeling.

Om de frequentie van detectie van HPV DNA in histologisch tumor-vrije lymfklieren te onderzoeken, is paraffine-ingebed weefsel van de linker obturator lymfklier van 50 patiënten met HPV 16 DNA in de primaire tumor onderzocht met behulp van PCR met de nieuw ontwikkelde HPV-16 specifieke 'primer set' (hoofdstuk 7). HPV-16 DNA werd aangetoond bij 7 van de 15 vrouwen met tumormetastase in andere lymfklieren, tegen 12 van de 35 patiënten die histologisch tumor-vrij waren in alle onderzochte lymfklieren. Aangezien slechts één van de 12 vrouwen met HPV-16 DNA positieve, histologisch tumor-vrije lymfklieren een tumorrecidief ontwikkelde, kan geen prognostische waarde worden gehecht aan de detectie van HPV-16 DNA in histologisch tumor-vrije lymfklieren van vrouwen met HPV16 DNA in de primaire tumor.

Samenvattend kan gezegd worden dat in alle serologische testen de prevalentie van antistoffen tegen HPV-16 E6 en E7 significant hoger was in cervixcarcinoom patiënten dan in controles en CIN patiënten. De resultaten van de antistof detectie met behulp van de E7 synthetisch peptide-ELISA of de op E6 en E7 eiwit gebasserde RIPA geven echter aan dat de prevalentie van antistoffen in cervixcarcinoom patiënten te laag is om bruikbaar te zijn

als diagnostische test. Dit neemt niet weg dat de aanwezigheid van antistoffen tegen HPV-16 E6 en E7 gerelateerd is aan clinico-pathologische parameters, zoals tumorgrootte en lymfkliermetastase, en aan overleving, hetgeen een prognostische waarde suggereert. Analyse van de antistofniveau's in patiënten direct na behandeling en gedurende follow-up heeft aangetoond dat in patiënten, die seropositief zijn vóór de behandeling, de antistofniveau's zijn gerelateerd aan het klinisch beloop. Deze bevinding, in samenhang met de relatie tussen seropositiviteit en een slechtere prognose, suggereert dat het waardevol is om patiënten, die seropositief zijn vóór behandeling, serologisch te vervolgen na behandeling. Gezien het relatief kleine aantal patiënten dat is onderzocht, is verder onderzoek naar de waarde van de antistofniveau's tegen HPV-16 E6 en E7 gedurende de follow-up van cervixcarcinoom patiënten noodzakelijk.

ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
APC	antigen-presenting cell
BPV	bovine papillomavirus
CA 125	cancer antigen 125
CEA	carcinoembryonic antigen
CIN	cervical intraepithelial neoplasia
CTL	cytotoxic T lymphocyte
E#	early (protein)
E2BS	E2-binding sites
E2RE	E2-responsive elements
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EV	epidermodysplasia verruciformis
FIGO	International Federation of Gynaecologists and Obstetricians
HPV	human papillomavirus
HSV	herpes simplex virus
IFN	interferon
IL	interleukin
kbp	kilo basepairs
L#	late (protein)
LAMP	lysosomal-associated membrane protein
LCR	long control region
MHC	major histocompatibility complex
NCR	non-coding region
NK cells	natural killer cells
ORF	open reading frame
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PV	papillomavirus
SSC-ag	squamous cell carcinoma antigen
TNF	tumor necrosis factor
TPA	tissue polypeptide antigen
TT-RIPA	transcription/translation-radioimmuno precipitation assay
URR	upstream regulatory region
VLP	virus like particle(s)

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CURRICULUM VITAE

De schrijver van dit proefschrift, Marc Baay, werd op 27 november 1965 geboren te Alkmaar. In 1984 werd het VWO diploma behaald aan het Nieuwe Lyceum te Bilthoven. De opleiding werd datzelfde jaar vervolgd met een studie Biologie aan de Landbouw Universiteit Wageningen (specialisatie celbiologie). Middels een tweetal afstudeervakken (Moleculaire Virologie en Genetica) en een stage bij the Institute of Animal Physiology and Genetics Research, Cambridge, UK, werd de studie afgerond. Eén maand voor het behalen van het doctoraalexamen werd hij aangesteld als toegevoegd wetenschappelijk medewerker bij de vakgroep Voedingsmiddelen van Dierlijke Oorsprong, faculteit Diergeneeskunde, Universiteit Utrecht. Aldaar werd in de periode juni 1990 tot en met maart 1992 onder leiding van Prof. Dr. J.H.J. Huis in 't Veld onderzoek verricht naar de serologische diagnostiek van *Salmonella enteritidis* bij pluimvee. In april 1992 werd de overstap gemaakt naar een Assistent in Opleiding positie bij de vakgroep Dermato-venereologie (Prof. Dr. E. Stolz), faculteit Geneeskunde en Gezondheidswetenschappen, Erasmus Universiteit Rotterdam. Via een detachering werd aan het Diagnostisch Centrum SSDZ te Delft onder leiding van Dr. P. Herbrink het onderzoek uitgevoerd dat in dit proefschrift is beschreven.

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