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**Presented by:**

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# **Characterization of B-Cells Epitopes on HPV Capsid Proteins**

**Referees**

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The present cumulative thesis was performed under the supervision of Prof. Dr. Lutz Gissmann and Dr. Michael Pawlita during the period from April 2004 to November 2007 in the German Cancer Research Center (DKFZ, Heidelberg, Germany), Institute for Applied Tumor Virology, Research Program "Infection and Cancer"

**Publications included in this cumulative thesis and contributions of Raeda Rizk to them:**

### **Publication 1**

Reactivity pattern of 92 monoclonal antibodies with 15 Human papillomavirus types  
**Raeda Z. Rizk**, Neil D. Christensen, Kristina M. Michael, Martin Müller, Peter Sehr, Tim Waterboer, Michael Pawlita.

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**Contribution:** 75%, all experiment and related analysis were performed by Mrs. Rizk. The draft paper was written by Mrs. Rizk including several correction steps.

### **Publication 2**

Absence of SV40 antibodies or DNA fragments in pre-diagnostic mesothelioma serum samples

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**Contribution:** (15%) Mrs. Rizk developed the antibody blocking assays used in the experiments, an essential contribution to the publication.

### **Publication 3**

Humoral immune response in women with high grade cervical intraepithelial neoplasia (CIN II/III) after vaccination with HPV16 L1/E7 chimeric-virus-like particles (cVLP)

**Raeda Z. Rizk**, Kristina M. Michael, Tim Waterboer, Lutz Gissmann, Andreas Kaufmann, Michael Pawlita. Manuscript will be submitted to an international journal for publication.

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*To*

*Basem, Yara, Hasan, Father and Mother*

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## SUMMARY

Human Papillomavirus (HPV) are considered as the major etiologic cause for a variety of benign and malignant epithelial lesions. The so-called high risk-HPV types are related to cervical cancer, while low-risk types are associated with benign mucosal lesions only. HPV types 16 and 18 are the most frequently found types in cervical cancers. Antibodies to HPV major capsid protein, L1, can develop in response to natural HPV infection as well as after vaccination. In contrast, antibodies to early, non-structural proteins, mainly E6 and E7 which are consistently expressed in HPV-transformed cells, have been found to be strongly associated with invasive carcinoma. Virus-like particles (VLP) which mimic morphologically and immunologically the native virions have been used so far as standard serological antigens.

This PhD thesis had two main objectives, first to characterize the bacterially expressed, affinity-purified glutathione S-transferase fusion proteins (GST) as alternative antigens for serology in terms of displayed epitope repertoire, using a panel of 92 VLP-specific monoclonal antibodies (mAb) generated against 9 mucosal alpha papillomavirus types. The second objective was to analyze in details the natural as well as HPV vaccine-induced immune response in patients with cervical intraepithelial neoplasias (CIN II/III). Additionally, an antibody blocking assay based on Maltose binding protein (MBP)-fusions was established to analyze the type-specificity of HPV antibodies.

It is shown here that GST-L1 fusion proteins display a broad variety of epitopes and thus are well suited for detection of human HPV antibodies. Cross-reactivity is associated with linear as well as conformational epitopes and can be intra- and/or interspecies specific and follows the phylogenetic relationships only loosely. Neutralizing epitopes are always conformational and mostly, but not always type-specific. The established MBP-16L1 allowed blocking the binding of mono-specific as well as cross-reactive antibodies to GST-16L1 but not that of specific antibodies to other HPV types.

Vaccination of patients with cervical intraepithelial neoplasias with HPV16L1/E7 chimeric VLP induced strong 16L1 specific and weaker 16E7 specific responses as well as L1 neutralizing antibodies. The response was positively correlated with vaccine dose the immune status before vaccination. Vaccination-induced cross-reactivity to both L1 and E7 was across species and the cross-reactive sera were non

cross-neutralizing for distantly related HPV. Chimeric VLP vaccination also induced antibody responses to other HPV16 early proteins suggesting that these proteins were released from the lesions in response to vaccination.

In conclusion, bacterially expressed GST fusion proteins are good candidates to be used as antigens in HPV serology and are also useful tools to define and characterize the complex patterns of conformational and linear cross-reactive epitopes.

## ZUSAMMENFASSUNG

Humane Papillomviren (HPV) sind der wesentliche ätiologische Faktor für eine Reihe gutartiger und bösartiger epithelialer Läsionen. Die sogenannten Hoch-Risiko HPV Typen sind mit Gebärmutterhalskrebs assoziiert, Niedrig(Low)-Risiko Typen dagegen nur mit gutartigen Schleimhautläsionen. Die HPV Typen 16 und 18 sind die häufigsten Typen in Gebärmutterhalskrebs. Antikörper gegen das Hauptkapsidprotein L1 können sich nach natürlicher Infektion aber auch nach Impfung entwickeln. Dagegen sind Antikörper gegen die frühen, Nichtstrukturproteine E6 und E7, die konsistent in HPV-transformierten Zellen exprimiert werden, stark assoziiert mit invasivem Krebs. Virus-ähnliche Partikel (VLP) ahmen morphologisch und immunologisch native Virionen nach und sind bisher als Standardantigen für HPV Serologie verwendet worden.

Die beiden wesentlichen Ziele dieser Dissertation sind: (i) Bakteriell exprimierte, affinitätsgereinigte Glutathion-S-Transferase (GST) Fusionsproteine sollten bezüglich des Epitoperepertoires als alternative Serologieantigene charakterisiert werden unter Verwendung von 92 VLP-spezifischen monoklonalen Antikörpern, die gegen 9 mukosale Alpha-Papillomavirus Typen entwickelt worden sind. (ii) Die natürliche wie die durch HPV-Impfung induzierte humorale Immunantwort sollte in Patientinnen mit Gebärmutterhalskrebs-Vorstufen (CIN II/III) detailliert beschrieben werden. Weiterhin wurde ein Antikörper-Blockierungstest basierend auf Fusionsproteinen mit Maltose-Bindungsprotein (MBP) entwickelt für die Analyse von typspezifischen HPV-Antikörpern.

Hier wird gezeigt, dass GST-L1 Fusionsproteine eine breite Epitope-Palette präsentieren und so gut geeignet sind humane HPV-Antikörper zu bestimmen. Kreuzreaktivität ist assoziiert mit linearen und konformationellen Epitopen, sie kann intra- und/oder interspezies spezifisch sein und folgt nur wenig den phylogenetischen Verwandtschaften. Neutralisierende Epitopes sind immer konformationell und meistens, aber nicht immer typspezifisch. Mit dem Fusionsprotein MBP-16L1 konnte sowohl die Bindung von mono-spezifischen als auch kreuzreaktiven Antikörpern an GST-16L1 gehemmt werden aber nicht die von für andere HPV-Typen spezifische Antikörpern an die entsprechenden Typen.

In Serien von CIN II/III Patientinnen, die im Rahmen einer klinischen Studie mit HPV16L1/E7 chimären VLP geimpft worden waren, konnte die Induktion einer

starken Antikörperantwort gegen 16L1 und schwächer gegen 16E7 und von neutralisierenden Antikörpern nachgewiesen werden. Die Immunantwort war positiv korreliert mit der Vakzinedosis und mit dem Immunstatus vor der Impfung. Durch die Impfung induzierte Kreuzreaktivität für L1 und E7 ging über Speziesgrenzen hinaus, aber Kreuzneutralisation mit entfernt verwandten Typen wurde nicht beobachtet. Impfung mit chimären VLP induzierte auch Antikörper gegen andere frühe HPV-Proteine, was darauf hinweist, dass diese Proteine aus den Läsionen freigesetzt wurden im Rahmen der immunologischen Impfantwort.

Zusammengefaßt zeigt diese Dissertation, dass bakteriell exprimierte GST-Fusionsproteine gut brauchbare Antigene sind für die HPV-Serologie und nützliche Werkzeuge zur Definition und Charakterisierung komplexer Muster konformationeller und linearer kreuz-reaktiver Epitope sind.

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## **CHAPTER ONE**

# **1. Introduction**

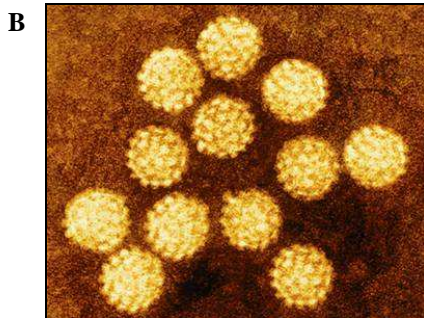
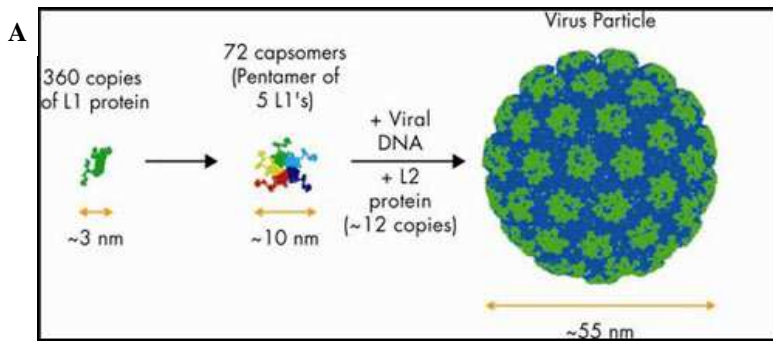
## **1.1 Human Papillomaviruses (HPV)**

Papillomaviruses (family *Papillomaviridea*) are widespread small double-stranded DNA viruses that infect the squamous epithelial cells of the skin and mucous membranes of human and a variety of animals and induce hyperproliferative lesions, e.g. warts. They are highly species- and tissue-specific viruses.

Certain types of human papillomaviruses (HPV) have recently been linked etiologically to cervical abnormalities including precancers, cervical cancer, warts, and recurrent respiratory papillomatosis. They are also found to be associated with subgroups of other malignancies such as squamous cell carcinomas of the anus, vulva, vagina, penis, and head and neck. Based on the frequency with which these types are found in cancers and precursor lesions, HPV can be divided into high-risk, intermediate-risk, and low-risk subtypes. Low-risk subtypes, such as HPV11 and 6 are associated with benign genital warts (condyloma acuminata), low-grade or benign cervical lesions (Schiffman & Castle, 2003). Intermediate and high-risk subtypes are associated with cervical dysplasia and invasive carcinoma.

## **1.2 Capsid structure**

HPV are small, non-enveloped icosahedral particles ~52-55nm diameter. There are 72 capsomers (60 hexameric + 12 pentameric) arranged on a T = 7 lattice. The icosahedral HPV capsid consists of 360 copies of L1 major structural protein (55 kDa) and approximately 12 copies of L2 (75 kDa) minor structural protein (Kieback & Muller, 2006) (Figure 1).



**Figure 1. Structure of HPV particle.**

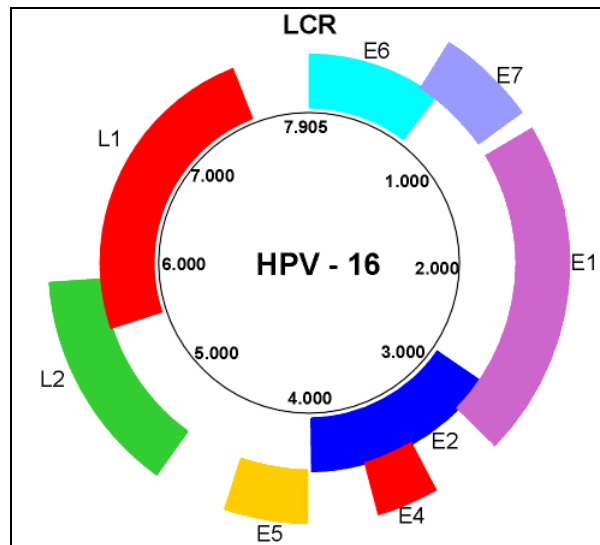
A) A single capsomere consists of 5 copies of L1 structural protein. 72 capsomeres with one copy of viral DNA and 12 L2 copies combine to form the infectious virus particle.

B) Electron microscopic picture of Papillomavirus particles. Source: [www.merckmedicus.com/.../hpvd/images/figure2.jpg](http://www.merckmedicus.com/.../hpvd/images/figure2.jpg)

### 1.3 HPV genome organization and major key proteins

Human papillomaviruses (HPV) contain a single molecule of double-stranded circular DNA of approximately 8 kb (Fig. 2) associated with cellular histones to form a chromatin-like substance (Modis *et al.*, 2002). HPV 16 genome is described here as a representative type, however, some differences may occur in other types, e.g the genome might slightly vary in size between different HPV types. HPV 16 genome can be divided into three regions, the noncoding long control region (LCR), or the upper regulatory region (URR), and the early (E) and late (L) gene region (protein encoding). The long control region of 400 to 1,000 bp contains cis-acting regulatory sequences and overlapping binding sites for many different transcriptional activators and repressors, including activating protein 1 (AP 1), and nuclear factor 1 (NF-I). The LCR regulates transcription from the early and late regions, and therefore controls the production of viral proteins and particles. The early region (about 4.5 KDa) is downstream of the LCR and contains six open reading frames, E1, E2 and E4—E7, and is involved in viral replication, transcription and cellular transformation. The late region (2.5 KDa) encodes the viral major and minor capsid proteins, L1 and L2 respectively. Both proteins are required late in the viral life cycle to encapsulate the virus (zur Hausen, 1996).





**Figure 2: Structure of HPV16 genome**

Source: <http://www.baclesse.fr/cours/fondamentale/7-carcino-virale/Virale-6.htm>

### ***HPV early proteins***

The HPV early (E) region in general encodes six open reading frames:

**E1** protein plays a role in initiating the replication of the viral DNA, maintenance of the viral episome as it encodes DNA-binding protein (Scheffner *et al.*, 1994). It is also essential in control of gene transcription. It is highly conserved among papillomaviruses.

**E2** protein is a DNA-binding protein and together with E1 is required for initiation of viral genome replication. It plays an important role in viral genome segregation during cell division by tethering the viral genome to the mitotic chromosomes (Skiadopoulos & McBride, 1998). It functions also in regulating the viral gene expression through a specific interaction with the early promoter found in the upstream regulatory region (Doorbar, 2006).

**E6** is a 16- to 19-KDa protein. It contains four Cys-X-X-Cys motifs which mediate the formation of two zinc finger structures (McMurray *et al.*, 2001). It binds to the p53 tumor suppressor protein and causes its destruction by the ubiquitin proteolysis pathway. This results in a loss of p53-dependent functions including G1 arrest and apoptosis. E6 is also a key oncoprotein in the development of cervical cancers as it compromises the effectiveness

of the cellular DNA damage response and allows the accumulation of secondary mutations to go unchecked.

**E7** is a 10- to 14-KDa phosphoprotein that has transforming activity and regulatory functions. It interacts with and degrades RB, which releases the transcription factor E2F from RB. The resulting high E2F activity might lead to apoptosis in E7-expressing cells (zur Hausen, 1996). E7 of high risk types bind additionally with other Rb-related proteins, such as p107, p130, and with the protein kinase p33cdk2. It also stimulates the S-phase genes, cyclin A and cyclin E and induces aneuploidy of the E7-expressing cells by inducing centriole amplification, which may contribute to tumorigenesis (Dyson *et al.*, 1989; Tommasino *et al.*, 1993). In high-grade cervical intra-epithelial neoplasia (CIN II/III) and cervical cancer, E7 and E6 are constitutively expressed in all layers of the infected epithelium.

Both E6 and E7 are expressed in addition to the other early proteins in the lower spinous layers, and they are considered as the predominant transforming proteins and therefore are potential targets for activated T cells in the development of strategies for immune responses.

**E5** protein is a hydrophobic trans-membrane protein that resides predominantly in the ER (endoplasmic reticulum). It interacts with and upregulates the trans-membrane domain of the EGFR (epidermal growth factor receptor) (Crusius *et al.*, 1997; Crusius *et al.*, 1998). E5 protein seems to be not necessary for the maintenance of the viral transformed phenotype as it is generally not detected in cervical cancers after viral genome integration (Munger *et al.*, 2004); however, it has been shown to prevent apoptosis following DNA damage.

**E4** protein seems to be one of the viral tools that contribute to the viral release process by binding and inducing the collapse of the cytokeratin network of the infected cells (Doorbar *et al.*, 1991; zur Hausen, 1996). E4 accumulates in the cell at the time of viral genome amplification, and its loss has been shown to disrupt late events in a number of experimental systems (Nakahara *et al.*, 2005; Peh *et al.*, 2004; Wilson *et al.*, 2005). It is located on the early region of the genome, but its expression pattern is more similar to the late proteins.

### ***HPV late proteins***

The HPV genome encodes two structural proteins, L1 and L2, which are expressed only late in infection in the differentiating keratinocytes (Stoler, 2000).

**L1** is the major capsid protein in the virus particle. It has a molecular weight of approximately 55 KDa and represents approximately 80% of the total viral proteins. Additionally it is the most highly conserved protein among all the papillomavirus proteins (de Villiers *et al.*, 2004). Serologically, neutralizing epitopes were found to locate on the exposed surface of L1 (Dillner, 1999). L1 has the intrinsic capacity to self-assemble into virus-like particles (VLP) (Hagensee *et al.*, 1994; Kirnbauer *et al.*, 1994; Zhou *et al.*, 1991).

**L2** is the minor capsid protein and it is thought that a single L2 molecule may be present in the centre of the pentavalent capsomeres at the virion vertices (Modis *et al.*, 2002; Trus *et al.*, 1997). L2 has a molecular size of approximately 72 KDa. Synthesis of L2 is initiated prior to synthesis of L1 in the terminally differentiating keratinocytes (Florin *et al.*, 2002). In the capsid, L2 may have a variety of functions possibly including binding of a secondary receptor, nuclear localization, or binding to the viral genome (Day *et al.*, 1998; Zhou *et al.*, 1994). It is essential for the infectivity of pseudovirions and probably also virions, but its exact role in papillomavirus infection is still unclear.

### **1.4 Classification**

Papillomaviruses are very diverse and they have a high degree of species specificity as there are no known examples of natural transmission of HPV to other species.

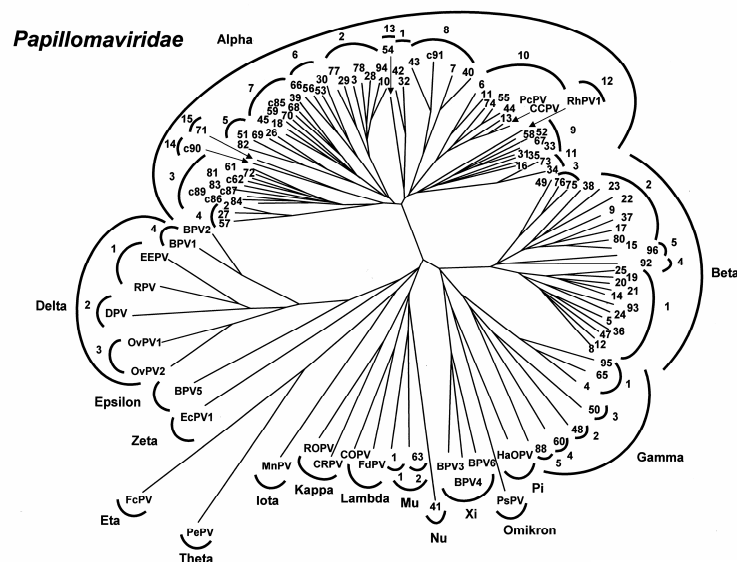
Papillomaviruses also display a marked degree of cellular tropism. Specific viral types appear to have a preference for either cutaneous or mucosal types.

So far, more than 100 HPV types have been fully characterized by cloning and complete sequencing of their genomes. Classification of HPV is based on the L1 open reading frame nucleotide sequence (Figure 3). HPV of the same genus show at least 60% sequence identity, those of the same species at least 70%, those of the same type at least 90%, those of the same subtype at least 98% and those of the same variant >98% (de Villiers *et al.*, 2004). The two main HPV genera are the alpha and beta papillomaviruses, encompassing approximately 90% of currently characterized HPVs. HPV of the genus alpha (12 species) mostly infect anogenital and oral mucosa, however, it also includes three species with cutaneous viruses such as HPV 2, which cause common skin warts and benign genital lesions (de Villiers *et al.*,

2004). Beta papillomaviruses are typically associated with inapparent cutaneous infections, but in immunocompromised individuals and in patients suffering from the inherited disease EV (epidermodysplasia verruciformis), these viruses are discussed to become associated with the development of non-melanoma skin cancer (Harwood & Proby, 2002; Pfister, 2003).

The remaining HPV come from three genera (Gamma, Mu and Nu) and generally cause cutaneous papillomas and verrucas that do not progress to cancer.

Approximately 15 high-risk types are known (most frequent types 16 and 18) (Munoz *et al.*, 2003) and they belong to genus alpha, species 5, 6, 7, 9 and 11 (Bosch & de Sanjose, 2003; Clifford *et al.*, 2003; zur Hausen, 2002). HPV16 is the most prevalent high-risk HPV in the general population, and is responsible for approx. 50% of all cervical cancers.



**Figure 3. Phylogenetic tree of 118 papillomavirus types based on homology of L1 ORF. Modified from de Villiers *et al* 2004.**

## 1.5 HPV life-cycle and pathogenicity

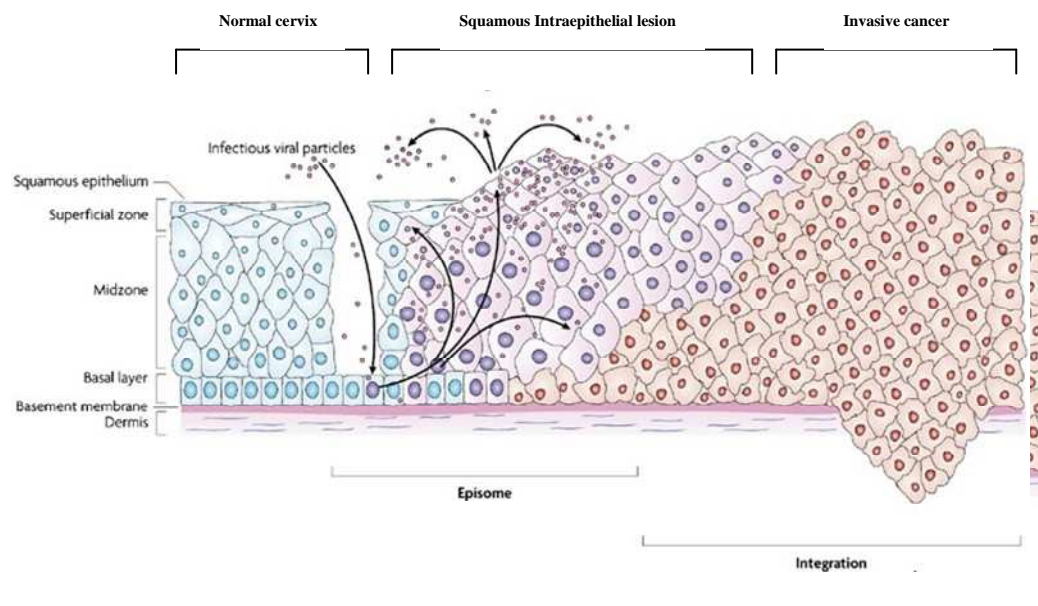
Viral infection is exclusively restricted to the basal keratinocytes, however, high level expression of viral proteins and viral assembly occur only in differentiating keratinocytes in the stratum spinosum and granulosum of squamous epithelium (Figure 4) (Peh *et al.*, 2002; Woodman *et al.*, 2007). The viral life-cycle has both a non-productive and a productive stage (Figure 4). The non-productive stage starts when the virus particle penetrates the epithelium via a microabrasion and infects the epithelial stem cells which are located in the basal epithelial cell layer. There, the virus establishes its DNA in the host cell episomally and maintain the genome copy number (50 to 100 copies per cell) (Chow & Broker, 1994; Durst *et al.*, 1985; McMurray *et al.*, 2001) by the help of the cellular DNA replication machinery and the expression of the viral early proteins E1 and E2.

When the infected cells undergo division, a subset of the daughter cells remain part of the basal epithelium, while the other cells leave the basement membrane to stratify and differentiate. During this process, the productive stage is started, as the virus amplifies its genome to a higher copy number, express the late genes, encapsidate the genome and finally the new viral progeny is produced.

In the majority of advanced lesions and invasive tumors, HPV is integrated into the host genome. E6 and E7 transcripts from integrated HPV genomes have increased stability compared with episomally derived viral mRNA, and there is an increased expression in more severe lesions and cancers. Following integration, continued E6 and E7 activity prolongs the cell cycle, leading to the loss of effective DNA repair mechanisms providing the opportunity for the accumulation of genetic changes in a multistep process resulting in the development of cancer. E6 and E7 are independently able to immortalize various human cell types in tissue culture, but efficiency is increased when they are expressed together.

When the vegetative virus replication is initiated, E6 and E7 proteins bring the differentiated cells back into S-phase. This leads to hyperproliferation of cells and thus to the typical macroscopic features of a papillomavirus infection, i.e. the formation of a wart.

The replication cycle (the time from infection to release of virus) usually takes a long time; about 3 weeks; since this is the time the basal keratinocyte needs to complete differentiation and desquamate. The time between infection and the appearance of lesion varied from weeks to months (Koutsky, 1997; Oriel, 1971; Schneider & Koutsky, 1992).



**Figure 4. HPV life-cycle and pathogenesis.** Human papillomavirus (HPV) is thought to access the basal cells through micro-abrasions in the cervical epithelium. The early HPV genes *E1*, *E2*, *E4*, *E5*, *E6* and *E7* are expressed after the infection, and the viral DNA replicates from episomal DNA. In the upper layers of epithelium the viral genome is replicated further, and the late genes *L1* and *L2*, and *E4* are expressed. *L1* and *L2* encapsidate the viral genomes to form progeny virions in the nucleus which can then initiate a new infection. The progression of untreated lesions to microinvasive and invasive cancer is associated with the integration of the HPV genome into the host chromosomes, with associated loss or disruption of *E2*, and subsequent upregulation of *E6* and *E7* oncogene expression. Modified from Woodman *et al*, 2007.

## 1. 6 HPV and human diseases

Several cancers are so far partially attributed to the consequences of chronic viral or bacterial infections. Examples are *Helicobacter pylori* (gastric cancer), Epstein-Barr virus (EBV) (Burkitt's lymphoma, nasopharyngeal carcinoma and Hodgkin's disease), Hepatitis B and C (hepatocellular carcinoma), human herpes virus type 8 (Kaposi's sarcoma) (zur Hausen, 1999).

Most HPV such as types 1, 2, 3 or 4 preferentially infect cutaneous skin and usually cause only benign disease (e.g. plantar, common warts), HPV -5, -8 and related types cause skin lesions in the rare genetic disease epidermodysplasia verruciformis, which in 40% of the patients progress to cancer (Akgul *et al.*, 2006; Favre *et al.*, 1998a; Favre *et al.*, 1998b). Low-risk mucosal HPV types 6 and 11 cause benign ano-genital condylomata acuminata, upper aerodigestive tract papillomas (most notably laryngeal papillomas), and conjunctival and ear canal papillomas (zur Hausen, 2002).

Infection by certain high-risk HPV types is strongly correlated with the development of malignant tumors as their DNA is detected in more than 99% of cervical cancers. World wide there are about 500.000 new cases each year commonly in 30-50-years old women and about 275.000 cervical cancer deaths, making it the second most common cause of cancer deaths in women (Parkin *et al.*, 2005). HPV 16 DNA is detected in >50% of all cervical tumors (Clifford *et al.*, 2003; Munoz *et al.*, 2003). The next most common HPV type associated with cancer (found in 10-14% of all cervical carcinomas) is HPV 18 followed by HPV types 31 and 45 which cause an additional 10 % of all cases (Bosch *et al.*, 2002). About 80% of cervical cancers occur in less-developed countries. This high proportion is mainly due to the lack of well-organized screening programs.

HPV infections are more frequent in immunocompromised individuals such as organ-transplant recipients (Sillman *et al.*, 1997) or HIV-infected patients (Ferenczy *et al.*, 2003; Palefsky, 2007). As only a small fraction of women infected with oncogenic HPV types will eventually progress to high-grade squamous interepithelial lesions (HSIL) and cervical cancer, additional risk factors including high parity, long-term use of oral contraceptives, smoking, and co-infection with other sexually transmitted agents are the most consistently identified environmental co-factors and are likely to influence the risk of progression from cervical HPV infection to HSIL and invasive cervical cancer.

## 1.7 Immune response to HPV

During the natural course of HPV infection, papillomaviruses in general are not very immunogenic and the natural immune response is very weak in comparison to most of other viral infections (Frazer, 1996). HPV persistence suggests that the virus uses several mechanisms that function to direct the host immune response away from the virus:

1. There is no sign of inflammation within a wart due to the inability of the keratinocytes to function as antigen-presenting cells and therefore there is no obvious danger signal to alert the immune system.
2. The immune response against L1 and L2 proteins is slow to appear and does not occur in all infected individuals as the virus delays the production of these immunogenic capsid proteins until the skin cells have terminally differentiated into squamous epithelium which is sloughed off and not accessible to the immune cells.
3. The viral early proteins E6 and E7 that mediate cell proliferation in the lower epithelial layers are expressed at very low levels.
4. Expressed E6 protein regulates E-cadherin expression and Langerhans cell density (Matthews *et al.*, 2003).
5. Expression of E5 in the basal layers of the epithelium early in infection, down-regulates MHC class I expression resulting in avoidance of attack by effector cytotoxic T-lymphocytes (CTL) (Zhang *et al.*, 2003).
6. Both E7 and E6 can down-regulate IFN-dependent innate immunity (Barnard *et al.*, 2000).
7. The infection does not cause a lytic infection as the infectious particles are produced only in the upper epithelial layers in cells that are eventually lost from the epithelial surface at the end of their life span.

### ***Humoral immune response***

Natural HPV infection leads to induction of slow and modest but measurable serum antibody response in most but not all (50-70%) of the infected individuals (Carter *et al.*, 1995; Carter *et al.*, 2000; Dillner, 1999). These antibodies are specific for the major capsid protein L1 and are suggested to be directed against conformational epitopes; neutralize the virus. Conformational epitopes are operationally defined as those displayed on the outer surface of the intact virus particle, VLP and capsomeres and are destroyed by heat/alkali denaturation. Epitopes presented by small synthetic peptides and denatured VLP are defined as linear epitopes, these can also be present on intact VLP preparations either because they are



surface-exposed on capsids or because the VLP preparation also contains some denatured L1. Neutralizing epitopes have also been identified in the L1 major capsid protein (Christensen *et al.*, 1994; Giroglou *et al.*, 2001; Rose *et al.*, 1998; White *et al.*, 1998; White *et al.*, 1999). Monoclonal antibodies (mAb) generated against VLP of different HPV types including 6, 11, 16, 18, 31, 33, 35, and 45 (Christensen *et al.*, 1996a; Christensen *et al.*, 1996b; Combita *et al.*, 2002; Ludmerer *et al.*, 2000) have been used in identifying these different epitope displayed by HPV capsid.

The presence of these L1-specific antibodies is long-lasting; however, it does not contribute to the clearance of the infection (Shah *et al.*, 1997). In animal experiments, these antibodies are able to protect animals against further infection with the same virus type (Kreider & Bartlett, 1981; Suzich *et al.*, 1995).

HPV-L1 specific antibodies are considered markers for past or current infection as they are more frequently detected in patients with persistent infections or precancerous lesions (Carter *et al.*, 2000; Nonnenmacher *et al.*, 1995). On the other hand, antibodies specific for early proteins (mainly E6 and E7 oncogens) are only associated with the onset of cervical carcinoma (Lehtinen *et al.*, 2003; Meschede *et al.*, 1998; Silins *et al.*, 2002; Zumbach *et al.*, 2000). Regarding the other early proteins (E1, E2 and E4), limited data is available on responses in patients with HPV-associated diseases.

### ***Cellular immune response***

In this kind of immune response, the key players are T-cells (T-helper and cytotoxic T-cells). It can be found both in patients with and without HPV-associated lesions. Recognition of HPV-infected cells and stimulation of T-helper 1 (Th1) cells (which elicit the production of CTL) is mediated by the presence of dendritic cells or Langerhans cells in the cervical epithelium (Niedergang *et al.*, 2004). The stimulated cytotoxic effector cells can attach the infected cells resulting in clearance of the naturally acquired HPV infection (Stern, 2004) and/or regression of existing HPV-associated lesions.

### ***Cross-reactions between different HPV types***

Antibodies formed after infection with one-type of HPV usually do not bind to other types of HPV. Additionally, prior infection with one HPV type does not appear to prevent infection with another related type, which might suggest that generally different types of HPV correspond to different serotypes, and that the immune response to one type of HPV does not protect against other types of HPV (Thomas *et al.*, 2000). However, HPV 6 and 11 are one

exception as they contain shared epitopes on their intact capsids (>92% amino acid identity in L1); antibodies formed after infection with HPV 11 may partially protect against infection with HPV 6 and vice versa.

During the sexually active period of life, infection with more than one HPV type can happen and therefore co-occurrence of several type-specific serum antibodies is a common phenomenon. The very long persistence of HPV-specific serum antibodies after HPV DNA has been cleared and the common mode of transmission of these viruses could explain this phenomenon (Konya & Dillner, 2001). On the other hand, cross-reactions due to shared epitopes between different HPV types are also often observed, e.g, HPV 6 and 11 or HPV 18 and 45. Based on this, a major serological problem is the difficulty in distinguishing type-specific antibody response to multiple HPV types from cross-reactivity (Table 1).

Detailed study of the immune response and its correlation with the pathogenesis of HPV-associated lesions is very important for a successful vaccine development that helps in prevention of new viral infection as well as in treatment of already occurred HPV-associated lesions.

Genus	Alpha							
Species	9					7		10
Type	16	33	35	52	58	18	45	6b
Seronegative	14	54	17	22	11	80	0	148
	1	131	0	39	0	93	52	271
	0	0	0	0	0	0	0	297
	0	57	0	50	0	65	12	179
	28	0	0	38	0	0	108	65
Mono-reactive	3521	143	94	173	8	286	74	190
	0	120	178	57	192	2546	0	204
	0	172	8	0	0	37	31	2271
	0	55	4051	131	197	33	12	269
	111	116	0	0	985	175	7	203
	0	408	0	0	0	0	0	217
Oligo-reactive	43	109	14	74	1105	86	35	1065
	2403	46	0	18	0	151	0	2079
	136	196	0	0	79	601	403	643
	136	196	0	0	79	601	403	643
	86	59	99	38	1007	43	0	616
	7	106	1229	64	15	57	31	653
Multiple positive	754	312	97	123	538	496	110	845
	1382	1019	840	266	595	2411	1151	1557
	8622	2032	1792	1152	1395	3541	2290	1739
	2274	845	593	199	64	1484	1695	593
	1442	1209	1236	538	208	331	197	430
	0	1350	1129	1644	1319	149	0	348

**Table 1. Patterns of serological reactivity (Luminex-based GST-L1 antibody assay) of human sera to different HPV types.** Colored numbers are positive signals (>300 MFI). Reactivity to more than one type is often observed.

## 1.8 Vaccines against HPV infections

Vaccines against HPV have been or are currently developed from one side in order to prevent infection (protection) and on the other side to cure the already infected women (therapy). In this context, the induction of immunity to HPV proteins L1 and to E6 and E7, respectively, plays a crucial role in the strategy against HPV infection and subsequent HPV-related precancerous and cancerous lesions. L1 protein is used to elicit a strong humoral immune response from the host making it a good target for protective vaccination. On the other hand, the presence of E6 and E7 throughout the precancerous and cancerous stages makes them ideal targets for immune therapeutic vaccine development.

### ***Prophylactic vaccines***

They are vaccines that target HPV capsid proteins and elicit antigenic reaction from the host through the humoral immune response by inducing virus-neutralizing antibodies. A successful vaccine should immunologically mimic the infections it aims to prevent and prime the adaptive immune system to recall specific effector functions to any future encounters with the infectious agents.

### ***Therapeutic vaccines***

Therapeutic vaccines target those individuals who are already infected by high-risk papillomaviruses and who may or may not have developed clinical signs of the infection. For a successful vaccine, the chosen target antigens should be expressed in every infected cell and the vaccine should promote regression of HPV-associated lesions by the induction of cellular immune responses. These responses include, CD8<sup>+</sup> CTL, and cytokines with direct (IFN- $\alpha$  and IFN- $\gamma$ ) or indirect (interleukin-1, IL-1 or IL-2) effects on virus-infected cells (Frazer, 2004). E6 and E7 oncoproteins expressed in cervical cancer cells have been found to induce CTL responses in infected patients and specific antibodies in a proportion of patients with cervical carcinomas, so they are considered as the most suitable prime target antigens for immune therapy. Additional targets are the E1 and E2 proteins associated with viral replication/transcription (Jansen & Shaw, 2004).

### ***Clinical vaccination trials***

Several pre-clinical vaccination studies in animal models have been conducted. However, using the inactivated virions (when available, as there is no easy source for them) as vaccines was tricky and not suitable to be used in humans due to the possibility of introducing the viral oncogenes to the recipient's body. Alternatively, VLP were used as vaccines. These trials showed that VLP were protective both against cutaneous warts induced by the cottontail rabbit papillomavirus (CRPV) (Breitburd *et al.*, 1995; Jansen *et al.*, 1995) and also against mucosal warts induced by the canine oral papilloma virus (COPV) (Suzich *et al.*, 1995) and the oral bovine papilloma virus 4 (BPV 4) (Kirnbauer *et al.*, 1996). In these models, the vaccines used induced high-titers of serum-neutralizing antibodies and protected against experimental challenge with the infectious virus.

In humans, several randomized clinical vaccination studies based on L1-VLP of the high and/or low risk HPV types have been completed and some are still ongoing. The first HPV 16 VLP (Harro *et al.*, 2001), HPV 11 VLP (Evans *et al.*, 2001), HPV 16/11 (Fife *et al.*, 2004) and HPV 18 (Ault *et al.*, 2004) phase I vaccine trials showed that the vaccines were well tolerated and highly immunogenic even when administered without adjuvant and that the antibody titers in the immunized subjects was at least 40-folds higher than in natural infections. Several clinical phase II and III randomized placebo-controlled trials were also conducted. The vaccines used were either monovalent HPV 16 (Koutsky *et al.*, 2002; Mao *et al.*, 2006; Poland *et al.*, 2005), bivalent HPV 16/18 (Harper *et al.*, 2004; Harper *et al.*, 2006), or quadrivalent HPV 16/18/6/11 VLP (Villa *et al.*, 2005; Villa *et al.*, 2006a). The vaccination results showed that these vaccines were well tolerated, highly immunogenic as they induced type-specific neutralizing antibodies, provided complete protection against persistent type-specific infections and HPV associated diseases and induced long-term immunity for up to 4.5 years (Harper *et al.*, 2006; Villa *et al.*, 2006b). Besides the induction of antibodies, these vaccines were able to induce L1-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses and Th1 and Th2 cytokines (Pinto *et al.*, 2005). Two prophylactic vaccines are now commercially available, Glaxo Smith Kline's (GSK) bivalent vaccine named Cervarix<sup>TM</sup> and the Merck quadrivalent vaccine Gardasil<sup>TM</sup>. Phase III trials of these two vaccines revealed that they were highly efficacious in preventing HPV 16/18 related CIN II/III and adenocarcinoma in situ (AIS) (Ault, 2007; FUTUREII, 2007; Garland *et al.*, 2007; Paavonen *et al.*, 2007) and high grade vulval and vaginal lesions associated with HPV 16 or HPV 18 infection (Joura *et al.*, 2007).

A successful control for HPV-associated lesions and cancer would be to develop a vaccine that has a prophylactic as well as therapeutic property; able to induce neutralizing antibodies and a T-cell mediated immune response. To this end, chimeric VLP (cVLP) were established as alternative to VLP. CVLP are generated by the fusion of E7 protein to L1 or L2 (Muller *et al.*, 1997; Wakabayashi *et al.*, 2002) and when used for immunization in mice, they were able to induce L1-specific and neutralizing antibodies, to elicit an E7-specific cellular immune response and to prevent the growth of E7-positive cells in mice (Greenstone *et al.*, 1998; Nieland *et al.*, 1999; Schafer *et al.*, 1999). *In vitro* vaccination in human (using purified peripheral blood lymphocytes, PBL) revealed also the ability of the cVLP to trigger a specific T-cell immune response (Kaufmann *et al.*, 2001). Results of a recently published randomized, double-blind, placebo-controlled phase I/II clinical vaccination trial with HPV16 L1/E7 cVLP targeting patients with HPV 16 positive CIN II/III demonstrated an evidence for the safety of the vaccine and a non significant-trend for clinical efficacy. The trial showed that

the vaccine is able to induce L1 and E7 specific antibodies and also a cellular immune response (Kaufmann *et al.*, 2007).

## **1.9 HPV serology**

Serological assays are used to detect systematic immune response developed upon infection with HPV. However, the development of such assays faces some limitations including the large number of HPV types associated with different diseases and difficulties in isolating or producing sufficient quantities of infectious virions in cell cultures or *in vivo*.

### ***Developments of antigens used in serology***

Different antigens have been used so far in different established assays to overcome these limitations.

## **1. VLP**

Different expression systems to over-express the L1 capsid protein and generation of VLP have been used including vaccinia virus (Hagensee *et al.*, 1993), plasmid vectors (Pastrana *et al.*, 2004) or baculovirus (Kirnbauer *et al.*, 1992) in yeast (Sasagawa *et al.*, 1995), mammalian, or insect cells (Le Cann *et al.*, 1994), respectively.

VLP mimic the natural virions morphologically and immunologically and they display immunodominant conformational epitopes (Christensen *et al.*, 1996a; Christensen *et al.*, 1996b; Christensen *et al.*, 2001) and are able to generate high titers of type-specific neutralizing antibodies (Le Cann *et al.*, 1994; Schiller & Lowy, 1996).

## **2. Fusion proteins**

It is possible to achieve a favourable yield of recombinant proteins in *E.coli*, but obtaining the protein in a soluble, biologically active form was a major challenge so far. In the meantime, fusion proteins have become of great importance in the biological researches with a wide range of applications. Examples of these worth to use fusion proteins are, thioredoxin (TRX) (LaVallie *et al.*, 1993), Protein A (Samuelsson *et al.*, 1994), ubiquitin (Power *et al.*, 1990), DsbA (Zhang *et al.*, 1998), glutathione S-transferase (GST) (Nygren *et al.*, 1994) and maltose-binding protein (MBP) (Pryor & Leiting, 1997). In the context of this thesis, the last two fusion proteins are of interest.

### ***Glutathione S-Transferase (GST)***

GST (26 KDa) is derived from the parasitic helminth protein of *Schistosoma japonicum*. Recombinant expressed GST fusion proteins can be expressed in bacteria (Smith & Johnson, 1988), yeast (Lu *et al.*, 1997), mammalian cells (Rudert *et al.*, 1996) or baculovirus-infected insect cells (Beekman *et al.*, 1994). They could be purified from crude lysates by affinity chromatography on immobilized glutathione.

### ***Maltose-binding proteins (MBP)***

MBP is a periplasmic protein (40 KDa) encoded by the *malE* gene of *E.coli* K12 (Duplay *et al* 1988). It constitutes a part of the maltose/maltodextrin system of the bacteria which is responsible for the uptake and efficient catabolism of maltodextrins and used for the secretion of proteins into the periplasm (Blondel & Bedouelle, 1990). MBP can facilitate the solubility of over expressed fusion proteins in bacteria, especially eukaryotic proteins (Sachdev & Chirgwin, 1998). One-step purification of the MBP fused proteins is based on the strong affinity of MBP to cross-linked amylose. The MBP system is widely used in combination with a small affinity tag (Podmore & Reynolds, 2002).

### ***Serological assays***

Recent developments in HPV serological techniques include capture assays using bacterially expressed recombinant viral proteins (Sehr *et al.*, 2001; Sehr *et al.*, 2002) and the introduction of fluorescent bead-based technologies (Luminex) (Waterboer *et al.*, 2005; Waterboer *et al.*, 2006) which resulted in increased sensitivity and specificity of HPV antibody detection.

In this PhD work the following assays were applied. The detailed step by step description is explained in the individual contributions as cited below.

#### **1. VLP-capture ELISA**

So far, VLP-based ELISA is the standard technique to detect HPV L1 antibodies. It was first developed to screen for antibodies to HPV1 (Carter *et al.*, 1993) and HPV16 (Kirnbauer *et al.*, 1994). The assay was then applied for many other HPV types including Canine oral PV (CoPV) (Suzich *et al.*, 1995), Cottontail rabbit PV (CRPV) and bovine PV 1 (BPV 1) (Christensen *et al.*, 1996a), BPV4 (Kirnbauer *et al.*, 1996), HPV45 (Touze *et al.*, 1996), HPV6 and 11 (Touze *et al.*, 1998), HPV5 (Favre *et al.*, 1998b), HPV8 (Bouwes Bavinck *et al.*, 2000), HPV31, 33, 35, 18, 39 (Giroglou *et al.*, 2001), HPV59 (Combata *et al.*, 2002) and HPV15, 20 and 24 (Feltkamp *et al.*, 2003). The critical issue for VLP-based ELISA is the complexity of VLP production and the time consuming long purification procedure. Therefore, several attempts have been made to improve the specificity of the VLP-based antibody assays by capturing correctly folded antigen through mAb recognizing conformational L1 epitopes (Carter & Galloway, 1997; Wang *et al.*, 1997), through heparin (Wang X, 2005) or by competition assays, in which binding of a mAb to a conformational VLP epitope is inhibited by a test serum (Dias *et al.*, 2005; Palker *et al.*, 2001).

#### **2. Glutathione-S-transferase capture ELISA**

In this assay, recombinant proteins are over-expressed as GST fusion proteins in *E.coli* and affinity-purified directly on glutathione cross-linked to casein (GC) as capture protein on the ELISA plates (Sehr *et al.*, 2001; Sehr *et al.*, 2002). Fusion with the GST moiety occurs via the N-terminus of the viral over-expressed protein (Wilce & Parker, 1994), therefore the fused proteins are thought to present in a native conformation. The bound full-length fusion-protein can be monitored via an amino acid sequence (KPPTPPPEPET) from the terminal



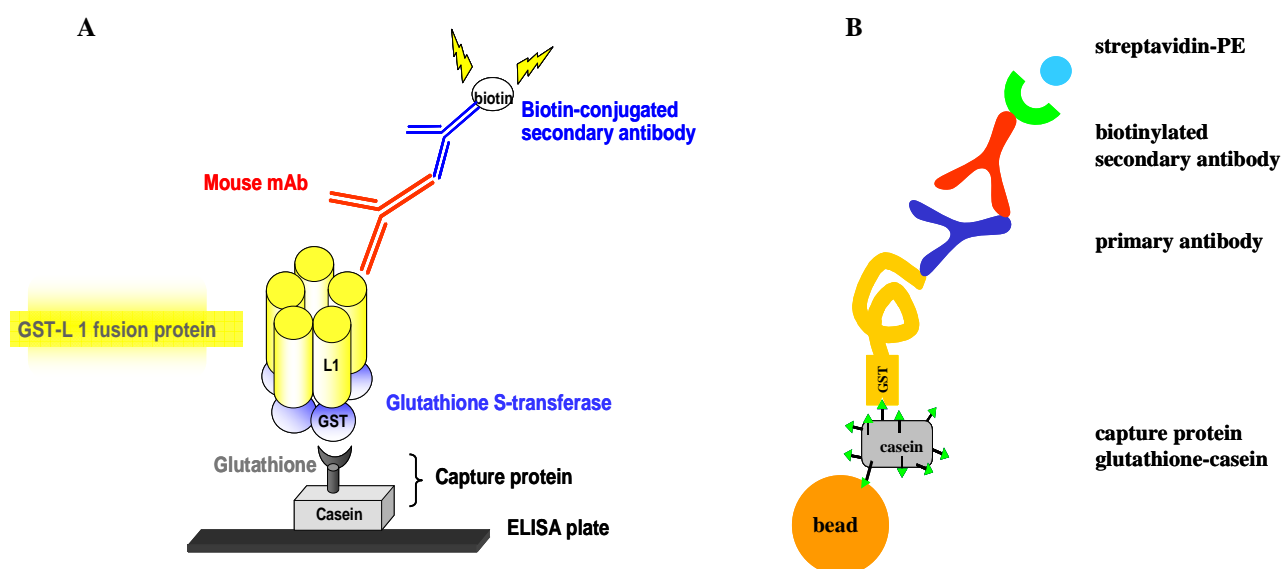
undecapeptide of the SV40 large T-antigen (tag) and fused here to the C-terminus of the HPV protein (Figure 5A). GST-capture ELISA is a very sensitive and highly specific and has been applied to a variety of antigens.

Both ELISA formats (using VLP or GST-fusion protein as antigens) were used in the **1<sup>st</sup> contribution** in a direct comparison to analyze and characterize the different epitope defined by 92 monoclonal antibodies.

### **3. Multiplex serology (Luminex)**

Seroepidemiological studies on prevalence and disease association with HPV require a method that allows a simultaneous analysis of large number of sera to many different antigens under the same working condition. Introduction of fluorescent bead-based technology (Luminex, Multiplex serology) (Waterboer *et al.*, 2005; Waterboer *et al.*, 2006) resulted in increased sensitivity and specificity of HPV antibody detection systems. The technology is based on using polystyrene beads (about 5.6 µm in diameter) filled with two different fluorescent dyes in different ratios (Figure 5B). There are 100 sets of these beads distinguished by their internal colour and could be loaded with different antigens. To be able to use GST-fusion protein in this assay, the carboxyl groups of the beads are coupled to the terminal amines of the Glutathione-casein. Incubation of the GC-beads with the antigens, sera and the detection reagents can be done in special 96-wells washing microtiter plates with filter bottoms (Millipore). The Luminex analyzer consists of a needle in which the loaded beads are lined up prior passing through the detection chamber, allowing the single particle to be measured directly. In the detection chamber, two lasers are used; the red laser is responsible for the classification of the beads according to their colours and the green laser analyzes the binding of the antibodies by exciting the reporter fluorescent label bound to each bead.

Multiplex serology is advantageous over the classical capture ELISA as the assay allows for screening of antibodies against up to 100 different antigens in a single sample using only a very little amount of the test serum (2µl/well). Luminex assay seems to be also more sensitive and specific in comparison to ELISA; it can detect the very weak ELISA signals but with less undesired background.



**Figure 5:** Schematic illustration for **A) GST-capture ELISA** , modified from *Sehr et al 2001* and **B) Luminex system**, modified from *Waterboer et al 2005*.

This system has been used in the **3<sup>rd</sup> contribution** to analyze the humoral immune response in HPV 16 L1/E7 cVLP vaccination study.

#### 4. HPV 16 antibody blocking assay

So far, VLP-based and GST-L1-based ELISA have been used successfully in large HPV epidemiological studies (Dillner, 1999), however, false positive cross-reactive signals might be obtained. These responses could be due to the fact that incorrectly folded VLP present in the antigen preparation used, expose cross-reactive epitopes that might compromise the type-specificity of the VLP-based assay (Wang *et al.*, 1997). On the other hand, GST-L1 fusion proteins were also found to present also the conformational and neutralizing epitopes as well as the linear (denatured) epitopes (Yuan *et al.*, 2001) which are thought to be responsible for cross-reactivity signals. The antibody blocking assay described here, based on soluble MBP-capsid protein fusions. It was initially developed for HPV L1 proteins to find out if soluble L1 protein of one HPV type specifically blocks cross-reactivity to another HPV type.

## 5. Neutralization assay

Neutralizing epitopes have been identified in the HPV L1 major capsid protein (Christensen *et al.*, 1994; Giroglou *et al.*, 2001; Rose *et al.*, 1998; White *et al.*, 1998; White *et al.*, 1999). So far, most of the serological analyses of responses to natural HPV infection relied on assays (such as ELISA) that are surrogates for neutralization (Dillner 1999), or antibody displacement assays (Opalka *et al.*, 2003; Yeager *et al.*, 2000). The problem of these assays is the fact that the immunodominant neutralization epitopes of HPV are genotype-specific. In ELISA, non-neutralizing and cross-genotype reactive antibodies might also be detected. Generally, HPV neutralization assays are troublesome and mostly lack sufficient sensitivity to reliably detect serum-neutralizing antibodies following natural infection (unpublished data). Many alternative systems were explored to develop an applicable HPV neutralization assay using athymic mouse xenografts (Kreider *et al.*, 1987), raft culture systems (Meyers & Laimins, 1994) or producing infectious HPV pseudovirions *in vitro* (Rodén *et al.*, 1996; Touze & Coursaget, 1998; Unckell *et al.*, 1997). The most recent *in vitro* neutralization assay is based on pseudovirions carrying a secreted alkaline phosphatase (SEAP) reporter gene (Buck *et al.*, 2005; Pastrana *et al.*, 2004). In this assay, the pseudovirion production main strategy is based on maximizing the production of the two capsid proteins L1 and L2 together with a target reporter plasmid in mammalian cells. L1 and L2 genes (normally have very low expression levels in cultured mammalian cells) with extensive codon modification are used to overcome the negative regulatory features of the wild-type open reading frame (ORF) (Schwartz, 2000). To generate a high copy number of the reporter plasmid, an SV40 origin of replication is inserted into the target plasmid. The pseudovirion is produced in cells (adenovirus-transformed human embryonic kidney cell line, 293TT) transfected with SV40 genome to express high levels of SV40 large T antigen (LT). Antibody-mediated pseudovirion neutralization is detected by a reduction in SEAP activity. The assay is explained in details in the 1<sup>st</sup> and 3<sup>rd</sup> contributions.

## **1.10 Aims of this thesis**

This thesis focused, on one hand on evaluating GST fusion proteins as antigens for serological assays and on the other hand on the analysis of HPV L1 epitopes recognized by experimentally and/or naturally induced antibodies. The three contributions included in this thesis had the following detailed specific aims.

### ***Contribution 1***

Using a panel of 92 monoclonal antibodies a study was conducted to:

1. evaluate glutathione *S*-transferase (GST)-L1 fusion proteins as ELISA antigens for detecting capsid specific antibodies against alpha papillomaviruses.
2. analyze the epitope repertoire (including type-specific, cross-reactive, conformational, linear and neutralizing epitopes) displayed by GST-L1 fusion proteins in comparison to VLP.

### ***Contribution 2***

The main objective was to establish a blocking assay based on soluble MBP fusion proteins to be able to differentiate between true infections and cross-reactions.

### ***Contribution 3***

The major aim of the analysis of a clinical cVLP (HPV 16 L1/E7) vaccination trial is to describe in detail the vaccine-induced antibody response including:

1. HPV 16L1 specific and neutralizing antibodies,
2. HPV 16E7 specific antibodies,
3. cross-reactive L1 and E7 antibodies and/or L1 cross-neutralizing antibodies.
4. specific antibodies to HPV 16 early proteins E1, E2, E4 and E6.

## 2. Major findings

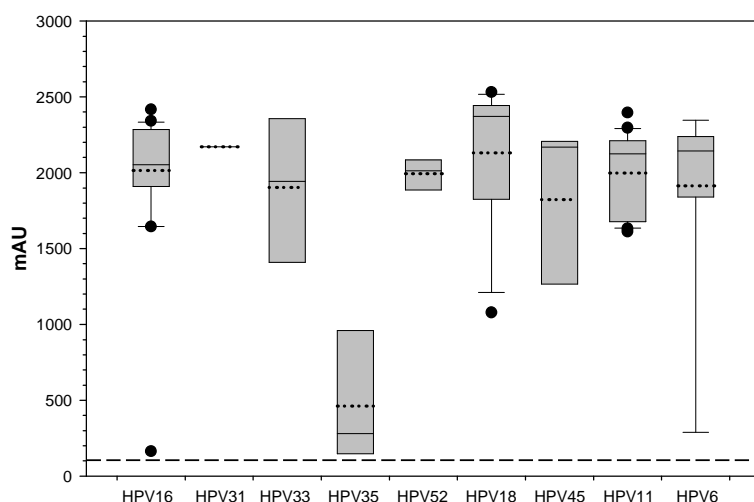
### 2.1 Analysis of reactivity pattern of 92 monoclonal antibodies with 15 Human papillomavirus types

In the first contribution (*Reactivity patterns of 92 monoclonal antibodies with 15 human papillomavirus types*) 92 VLP-specific monoclonal antibodies (mAb) generated against 9 mucosal alpha papillomavirus types of species 7, 9, and 10 were used to evaluate GST-L1 fusion proteins as ELISA antigens for detecting capsid-specific antibodies against alpha papillomaviruses in comparison to VLP, and to define which epitopes are type-specific or shared by several HPV types. L1 cross-reactivity patterns were determined with 15 different HPV types representing 6 species (alpha 1, 2, 4, 7, 9, and 10). Neutralization and cross-neutralization properties with HPV types 6, 11, 16, 18 and 45 were also investigated.

At 1:10 antibody dilution, eighty nine (97%) mAb were reactive with the specific HPV type used as immunogen ( $\text{mAU} \geq 110$ ). The positive absorbance (A) values varied between 340 and 2531 mAU (Figure 6). Although the remaining three mAb had estimated IgG concentration of  $>800$  ng/ml, but they were either very weakly reactive (*H6.C6* and *H35.O3*) or non-reactive (*H16.L4*) at all with their specific HPV GST-L1 protein. They all had  $A_{450} < 110$  at 1:90 dilution.

Further specificity analyses included titration of the different mAb against their specific immunogens. This step allowed also determination of antibody titer which reflects the antibody concentration in the different preparations. The end point antibody titer was defined as the last dilution giving a signal equal or greater 110 mAU.

All HPV 16 (except *H16.L4*) mAb were strongly reactive with GST-16L1 fusion protein and had  $A_{\text{max}}$  values above 1500 mAU. In tissue culture supernatants as antibody source, log end point titers varied from 2.2 to 6.2 (median 5.5). For the ascites monoclonal antibodies (*H16.U4* and *H16.V5*), the log antibody titers were 6.8 and 7.3 respectively. When excluding *H16.L4*, the  $A_{1/2\text{max}}$  values ranged from 823 (*H16.E70*) to 1209 (*H16.5A*) (mean 1043, median 1051, standard deviation (SD) 111) indicating about only 1.5 fold variation in the mAb concentration of the different supernatants.



**Figure 6. Reactivity of monoclonal antibodies with GST-L1 fusion proteins of the respective HPV type used for immunization.** 92 monoclonal antibodies generated against VLP of different 9 HPV types were tested at 1:10 dilution in GST-L1 capture ELISA. Only one HPV 31 specific mAb was tested. 89 of the mAb reacted with the specific type used as immunogen. The dotted short lines represent the mean of mAU for each mAb group. The dashed long continuous line represent the cut-off (110 mAU) used to define the reactive mAb.

Similarly, mAb against HPV 31 , 18 , 45, 33, 11, 52 and 6 reacted strongly with GST-L1 of the respective type (at 1:10  $A_{\max}$  greater than 1500) with log end point titers between 2.2 and 6.2 (median 4.8). The very high  $A_{\max}$  and the sigmoidal titration curves with steep slopes indicated high affinity of these antibodies to their immunogens.

The remaining six mAb (*H33.E12*, *H35.Q8*, *H35.H9*, *H35.N6*, *H45.N5* and *H18.A7*) showed  $A_{\max}$  values below 1500 for HPV 33 or 1100 mAU for the other types. Titers were between log 2.0 and log 4.3 (median 3.4) and the titration curves were flat.

Reanalysis of the 28 HPV16 mAb at a different day revealed a high reproducibility of the assay (the coefficient of determination of the linear regression of the absorption values was  $R^2=0.79$ ).

Of the 89 reactive mAb, 43 (48%) showed different inter- and /or intra-species cross-reactivity patterns while the remaining 46 (52%) were mono-specific. All HPV 31, 35, and 52 mAb were monotypic. The most frequent intra-species cross-reactivity among HPV 16 mAb

was with HPV 35 followed equally by HPV 31 and 58 and then HPV 33. HPV 52 was the least frequent cross-reactive type. The most frequent inter-species cross-reactive types were HPV 18 followed by HPV 45, 11, and 6. Reactivity to some alpha skin types was also observed. HPV 33 and 31 cross-reactive mAb, reacted only with closely related types (within  $\alpha 9$  species), except one single cross-reaction for HPV 31 mAb with HPV 32. Most frequent cross-reactivity to skin HPV types were observed among HPV 18 cross-reactive mAb. On the other hand, cross-reactivity to the mucosal high-risk of  $\alpha 9$  or to the low-risk of  $\alpha 10$  and  $\alpha 1$  were not frequent or were absent. For HPV 45 mAb, no inter-species cross-reactivity was observed. HPV 6 and 11 showed only rare and weak inter-species cross-reactions.

Thirty four (46%) mAb neutralized pseudovirions of the specific HPV types used as immunogens (HPV 16, 18, 45, 6 and 11) with end point neutralization titers ranging from log 1.7 to log 5.6. Only one cross-neutralizing mAb (*H6.L12*) has been reported. It neutralized HPV 6 as well as HPV 11 with only 0.3 fold difference in the log end point neutralization titer.

Retesting of mAb with the same immunogen (VLP) used in their production showed that all HPV 16 mAb and the four generated against other HPV types but cross-reacted with HPV 16 reacted in VLP-ELISA including *H16.L4* which was non-reactive in GST-L1 ELISA and is known to detect an epitope located within the N-terminus of L1 (Christensen 1996 and unpublished). Generally, the titers in VLP-ELISA and GST-L1 ELISA were similar for neutralizing and for monospecific mAb, whereas the GST-L1 titers were higher than the VLP-ELISA titers for the non-neutralizing and for the cross-reactive mAb. For mAb recognizing linear/denatured epitopes, the GST-L1 to VLP titer ratios were higher than for mAb recognizing conformational epitopes.

As a main objective of this study was to determine the epitope repertoire of GST-L1 fusion proteins, it was also interesting to characterize the type of epitopes recognized by the different mAb which reflects the epitope types that can be displayed by the GST-L1 fusion proteins. Epitope types of 28 mAb were defined in this study. Detection of epitope type was done by an immuno dot blot method using both native and denatured GST-L1 proteins.

Conformational epitopes were defined when a signal appeared only under native and not denatured conditions whereas denatured or linear epitopes were defined when a signal appeared under either only denatured or both native and denatured conditions. The epitopes

of five mAb (*H33.J3*, *H35.H9*, *H35.N6*, *H35.O3* and *H18.A7*) could not be defined as in at least two assays no signal under any conditions was detected even when using high concentration of the mAb.

## **2.2 Establishment of HPV16 antibody blocking assay**

This assay was initially established to distinguish antibody response to multiple HPV types from cross-reactivity.

HPV-16L1 protein fused to MBP was chosen, validated by ELISA and finally evaluated for suitability as a candidate to block epitopes displayed by GST-L1 fusion proteins used in serology. Anti-tag ELISA proved that MBP-16L1tag can react efficiently as well as GST-16L1 does with HPV 16L1 specific antibodies of different sources (mouse and human). This indicated that fusing MBP to HPV16L1 had no adverse effect on protein folding and therefore on presentation of the different epitopes (*see appendix 1, 2*).

Preabsorption of sera with MBP-16L1 lysate should specifically eliminate the specific reactivity to HPV 16 and any HPV 16L1-induced cross-reactivity to the very closely related HPV types of the same species (e.g HPV 31, 33, or 35) and also to other more distantly related types. Additionally, preabsorption with MBP-16L1 should not affect binding of antibodies specific for other types.

The blocking activity of MBP-16L1 was titrated and the assay was evaluated using HPV-reactive mAb as well as human sera.

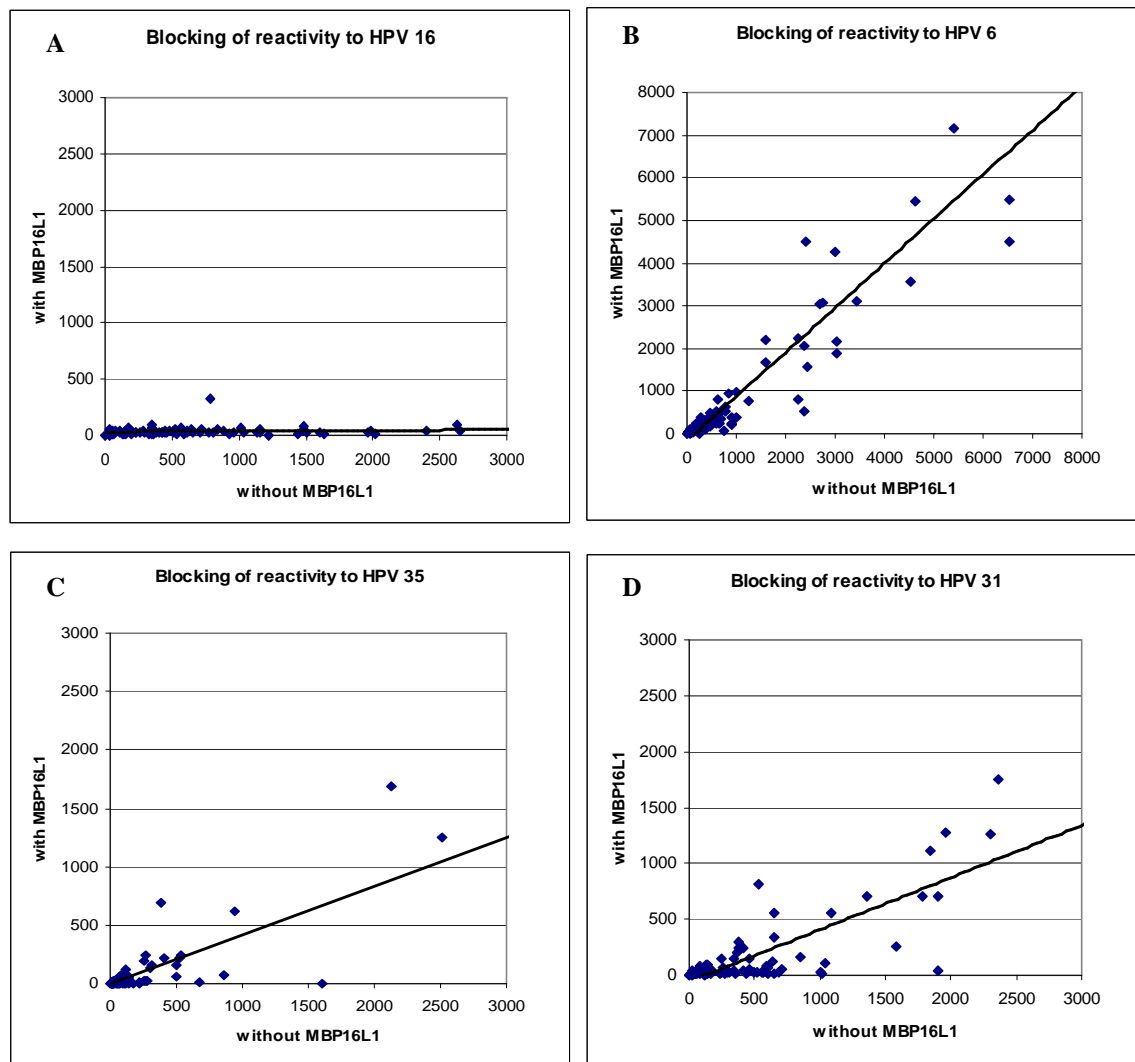
Pre-incubation of mAb or selected human sera with soluble MBP-16L1 fusion protein (2 mg/ml, *see appendix 3*) efficiently blocked binding (>80% reduction in reactivity) of HPV16 mono-specific and cross-reactive mAb/sera to GST-16L1. Reactivity of mAb/sera specific for other HPV (e.g HPV 6, 5 or 18) was unaffected (*see appendix 3, 4*).

The first application for the established blocking assay was successfully done using a large panel of human sera. These sera showed multiple positive signals to multiple different closely or distantly related HPV types. This analysis was not done by ELISA but instead by bead-based multiplex HPV serology as described earlier in section (2.3).

Pre-incubation of the sera with MBP-16L1 blocked their reactivity to GST-16L1 almost completely. Positive reactions for HPV types closely related to HPV16 e.g. HPV 31 and 35 were either completely or partially blocked. In case of HPV 31, 66% of the cross-reactions were completely blocked, while only 8% of the specific reactions were partially blocked (>50% reduction in MFI values). A similar picture was obtained for HPV 35 (60%



completely and 18% partially blocked). Sera showing positive signals to HPV types less strongly related to HPV16 (e. g HPV 6) retained their reactivities as only about 20% of the reactions were either completely or partially blocked.



**Figure 7 (continued).** Soluble MBP-HPV16L1 fusion protein blocks human HPV 16L1 (A) mono-specific and cross-reactive antibodies but not (B) HPV 6, (C) 35 or (D) 31 specific antibodies. Results are shown in Median Fluorescence Intensity (MFI) values.

The assay was successfully extended to HPV 6. MBP-6L1 blocking assay was applied to children sera of a Finnish HPV family study. The aim of this study is to investigate whether a previously observed HPV 6 and 11 responses in children in age of 6 month or older are type specific or are only due to cross-reaction with a non HPV infection. The preliminary data

suggested that these detected antibodies are different from the maternal antibodies transmitted to the children from their mothers. (*T. Enkirch, Diploma thesis 2007*).

In another application, the developed blocking assay principle was extended to polyomaviruses to analyze the specificity of putative human anti-SV40 (Simian Virus 40) antibodies in case control studies assessing by serology the controversially discussed association of SV40 with human tumors. See the **2<sup>nd</sup> contribution**.

### **2.3 Analysis of humoral immune response in human sera using GST-L1 fusion proteins (vaccination study)**

The third contribution in this thesis aimed mainly to analyze in details the humoral immune response to HPV antigens induced by vaccination with HPV16 L1/E7 chimeric virus-like particles (cVLP) using sera from a clinical vaccination trial with 36 participants, 12 placebo, 12 low-dose and 12 high-dose recipients (Kaufmann *et al.*, 2007). Analyses were done by multiplex serology as briefly described above and by *in vitro* neutralization assays based on HPV pseudovirions carrying a SEAP reporter gene (Buck *et al.*, 2005; Pastrana *et al.*, 2004).

As the participants were CIN II/III patients, it was not unexpected to detect a certain level of immune response at baseline (at week 0; W0). At this point, 21 and 6 participants had antibodies against HPV 16 L1 and E7, respectively, and 16 had HPV 16 neutralizing antibodies. None of the 12 placebo recipients showed any changes in the immune response; they did not seroconvert nor increase the reactivity (versus baseline) toward any of the antigens analysed.

All 24 women who received the vaccine showed an HPV16 L1 immune response already two weeks (W2) after receiving the first vaccine dose. The increase in response was strong as very high MFI values at 1:100 dilution as well as high endpoint dilution titers were reached. The increase in high-dose (250 µg) vaccinees was higher than in low-dose (75 µg) vaccinees (134- and 45-folds respectively). The geometric mean titer (GMT) among the high-dose group was more or less stable until W14; whereas in the low-dose group a continuous low rate increase in GMT was observed until the level of the high dose-group was reached at W14. At W24 the titers decreased 2.5- to 3-fold in both groups. The immune status of patients before vaccination correlates with the degree of increase in the immune response upon vaccination. In baseline seropositive participants GMT at W2 was 30 fold higher than in

baseline seronegatives and still 3-fold higher at W14. There was a linear relationship of endpoint titer (titers range 100 to 8100) and MFI values at 1:100 (MFI range 155 to 9344)

Vaccination leads also to generation of neutralizing antibodies. Eight of the vaccinees developed only a very weak neutralization response (border-line titers) over time, while 16 showed strong responses. The increase in the neutralization titer was observed already at W2, 1.8-fold stronger in the high-dose (GMT 1159) than in the low-dose group (GMT 661). At W14, the titer tripled among the high-dose and doubled in the low-dose recipients; but at W24 already decreased 5-fold and 3-fold, respectively, in both groups. Vaccinees baseline neutralization negative, at W2 reached a GMT of 283 only while the seropositives starting from 104 reached 487. The titers and the group difference did not change at W4 and W8, but with the increase at W14 the titer among baseline negatives became slightly higher than among the baseline positives, at W24 both fell to very similar low levels. No cross-neutralizing antibodies to either HPV 18 or 11 were detected

Cross-reactive antibodies to a variety of other HPV types than 16 including high- and low-risk mucosal alpha types, and different cutaneous alpha, beta, gamma, mu and nu types have also been detected. Cross-reactivity signals were identified as either those already existed at baseline and upon vaccination increased or those only induced after vaccination (at least 3-folds increase).

All of the vaccinees seroconverted to at least one HPV type other than HPV 16 irrespective of vaccine dose. However, HPV 16 specific titers were 243- and 81-folds greater than cross-reactive titers in high- and low-dose groups, respectively. The median time point of cross-reactivity seroconversions in patients of the high-dose group was W2 versus W4 in the low-dose group. At these weeks, the response plateau in most of the patients was reached. Frequency of cross-reactivity was generally higher in the high-dose than in the low-dose group with mean of 14 and 19 cross-reactive types in both groups, respectively. Cross-reactivity was more frequent among the alpha mucosal types followed by the alpha cutaneous and least frequent among the skin HPV types. All participants developed cross-reactive antibodies to HPV 18 and 45 although these types belong to a different and phylogenetically distant species.

All vaccinees (except one of the high-dose group) irrespective of the vaccine dose developed immune response to HPV16E7. The median time of serconversion in both vaccine groups

was W2. At this time point, the reactivity to HPV16 E7 in the high-dose group was 6.5-fold stronger than in low-dose recipients. The immune response in baseline seropositive participants was 3.4-fold higher than in baseline seronegatives.

Vaccination induced also E7 cross-reactive antibodies to at least one other type than HPV 16 in 13 vaccine recipients. However, the specific immune response to HPV16E7 was 2.5 stronger than to any of the cross-reactive types. Additionally, the time of seroconversions were late (after W4) in comparison to the specific HPV 16 E7 antibody response (at W2). Cross-reactivity was more frequent in the high-dose than in the low-dose group and followed the phylogenetic classification. Most frequent cross-reactions were detected with the closely related HPV types of the  $\alpha 9$  species. Cross-reactivity to types of  $\alpha 7$  or  $\alpha 10$  types was either absent or very rare in comparison to cross-reactivity observed for L1.

Antibodies to at least one of the other HPV 16 early proteins (E1, E2, E4 and E6) in 7/12 high-dose and 6/12 low-dose vaccinees were also detected. Responses were generally weak (MFI median 181, range 51-1217) and transient (some cross-reactions appeared only on a single time point and then disappeared). Seroconversion to any of these early proteins (W8 or later) occurred later than to HPV 16 E7 or L1 (W2). Antibodies to HPV16 E6 (36% of the vaccinees) were most frequent, followed by E4 (25%) and E2 and E1 (11% each).

### 3. General conclusions

The experimental data presented in this PhD thesis shed light on GST fusion proteins and their suitability to replace the traditionally used VLP as antigens in HPV serology assays. It also analyzed in details, the immune response in human after natural infections versus response due to vaccination. This allowed for the following conclusions:

From the mAb analysis study, we conclude the following

1. The bacterial expression system in comparison to other expression systems (e.g baculovirus or insect cells) allows easier analysis of many HPV types belonging to different species.
2. GST-L1 fusion proteins present most of the antigenic epitopes presented by VLP. They display the epitope repertoire of intact VLP (conformational) and also epitopes presented by denatured VLP (linear).
3. GST-L1 fusion proteins display neutralizing, type-specific as well as cross-reactive epitopes.
4. Cross-reactivity among HPV types can be inter-species, intra-species or mixed. It follows only loosely the phylogenetic grouping. Species-specific epitopes are either very rare or do not exist suggesting that the phylogenetic L1-based species definition may not define a serological unit.
5. Distantly related mucosal and skin alpha HPV types share conformational epitopes.
6. Cross-reactive epitopes are not necessarily cross-neutralizing.
7. Neutralizing epitopes are always conformational and are mostly type-specific. Cross-reactive epitopes are rarely neutralizing and if so they are rarely cross-neutralizing.
8. Soluble L1 protein of one HPV type can specifically block cross-reactivity to a different HPV type.

From the human vaccination study, we conclude the following:

1. HPV16L1/E7 cVLP vaccine is highly immunogenic.
2. CVLP can induce high titers of HPV 16L1-specific as well as high levels of E7-specific antibodies.
3. High titers of neutralizing antibodies can also be induced upon vaccination with cVLP
4. Luminex readout MFI values at 1:100 can be used as a surrogate to define or calculate the antibody and /or neutralizing titer of any serum.

5. The strength of the induced immune response depends on vaccine dose.
6. The immune status before vaccination correlated with the strength of the induced immune response.
7. CVLP vaccine induces wide L1 as well as E7 inter- and intra- species cross-reactivity patterns but not cross-neutralizing antibodies for distantly related types.
8. Cross-reactivity follows loosely the phylogenetic classification and the higher the vaccine response the more cross-reactivity is induced.
9. CVLP vaccine might have a probably immune reaction-induced cytopathic effect on cells and cause the release of other early proteins E6, E1, E2, and E4 present in the CIN cells.

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## **CHAPTER TWO**

## **1<sup>st</sup> Contribution**

### **Reactivity pattern of 92 monoclonal antibodies with 15 Human papillomavirus types**

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## Reactivity pattern of 92 monoclonal antibodies with 15 human papillomavirus types

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Most anti-human papillomavirus (HPV) capsid antibody assays are based on virus-like particles (VLP). We evaluated glutathione *S*-transferase (GST)–L1 fusion proteins as ELISA antigens for determining type specificity and cross-reactivity of 92 VLP-specific monoclonal antibodies (mAb) generated against nine mucosal alpha papillomavirus types of species 7, 9 and 10. The antibody panel included 25 new mAb, and 24 previously published mAb are further characterized. We determined the cross-reactivity patterns with 15 different HPV types representing 6 species (alpha1, 2, 4, 7, 9 and 10) and neutralization and cross-neutralization properties with HPV types 6, 11, 16, 18 and 45. Eighty-nine (97%) of the antibodies including 34, 71 and 14 recognizing neutralizing, conformational and linear epitopes, respectively, reacted with the GST–L1 protein of the HPV type used as immunogen, with log titres ranging from 2.0 to 7.3. Of these 89 antibodies, 52% were monotypic, 20% showed intra-species and 28% inter-species cross-reactivity. Log neutralization titres to the immunogen HPV ranged from 1.7 to 5.6. A single cross-neutralizing mAb (H6.L12) was found. ELISA titres were always higher than neutralization titres. All neutralizing epitopes were conformational and mostly type-specific. Our data show that bacterially expressed, affinity-purified GST–L1 fusion proteins display a broad variety of epitopes and thus are well suited for detection of HPV antibodies. Cross-reactivity is associated with linear as well as conformational epitopes. Distantly related mucosal and skin alpha papillomaviruses share some conformational epitopes and the phylogenetic L1-based species definition may not define a serological unit since no species-specific epitope was found.

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## INTRODUCTION

Human papillomaviruses (HPV) are small (about 55 nm in diameter) non-enveloped DNA tumour viruses with a tropism for squamous epithelia. The icosahedral protein capsid is composed of 72 capsomeres (pentamers of the major capsid protein L1) and contains a single molecule of about 8 kbp closed circular double-stranded DNA.

So far, more than 100 HPV types have been fully characterized by cloning and complete sequencing of their genomes. Classification of HPV is based on the major capsid protein L1 open reading frame nucleotide sequence: HPV of the same genus show at least 60% sequence identity, those of the same species at least 70%, those of the same type at least 90% and those of the same subtype (variants) at least 98% (de Villiers *et al.*, 2004). HPV of the genus alpha (15 species) mostly infect anogenital and oral

mucosa, some can additionally (species 2 and 8) or exclusively (species 4) infect the skin. Many alpha HPV can induce benign genital or common skin warts, while so called high-risk types (most frequently HPV types 16 and 18) (Munoz *et al.*, 2003) belonging to species 5, 6, 7, 9 and 11, can induce intraepithelial neoplasia, the precursor of cervical cancer (Bosch & de Sanjose, 2003; Clifford *et al.*, 2003; de Villiers *et al.*, 2004).

Upon infection with HPV, serum antibodies to L1 protein can develop (Dillner, 1999; van Doornum *et al.*, 1998; Wang *et al.*, 1996; Wideroff *et al.*, 1999). This immune response is highly type-specific (Carter *et al.*, 1996, 2000; Giroglou *et al.*, 2001; Wang *et al.*, 1997) and persists for years (Carter *et al.*, 2000; Dillner, 1999). L1 antibodies are considered markers for current and past infection (Carter *et al.*, 1996; Carter & Galloway, 1997; Kirnbauer, 1996) and are weakly associated with cervical cancer (Van Doornum *et al.*, 2003). In contrast, antibodies to the early oncoproteins E6 and E7 that are consistently expressed in HPV-transformed cells are strongly associated with cervical

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carcinoma (Lehtinen *et al.*, 2003; Meschede *et al.*, 1998; Silins *et al.*, 2002; Zumbach *et al.*, 2000).

Analysis of HPV humoral immune responses faces some limitations due to the large number of HPV types associated with the different diseases and to difficulties in producing sufficient quantities of infectious virus particles. As an alternative to virion production, efforts were done to reproduce the antigenic properties of virions by virus-like particles (VLP). VLP are formed spontaneously after overexpressing L1 by vaccinia virus (Hagensee *et al.*, 1993), plasmid vectors (Pastrana *et al.*, 2004) or baculovirus (Kirnbauer *et al.*, 1992) in yeast (Sasagawa *et al.*, 1995), mammalian, or insect cells (Le Cann *et al.*, 1994), respectively. VLP resemble papillomavirus virions in morphology and display conformational and neutralizing epitopes (Christensen *et al.*, 1996a, b, 2001; Le Cann *et al.*, 1994). VLP-based ELISA (ELISA) is the standard technique in HPV serology. It was developed to screen for antibodies to HPV1 (Carter *et al.*, 1993), HPV16 (Kirnbauer *et al.*, 1994), canine oral PV (CoPV) (Suzich *et al.*, 1995), cottontail rabbit PV (CRPV) and bovine PV 1 (BPV 1) (Christensen *et al.*, 1996a), BPV4 (Kirnbauer *et al.*, 1996), HPV45 (Touze *et al.*, 1996), HPV6 and 11 (Touze *et al.*, 1998), HPV5 (Favre *et al.*, 1998), HPV8 (Bouwes Bavinck *et al.*, 2000), HPV31, 33, 35, 18, 39 (Giroglou *et al.*, 2001), HPV59 (Combata *et al.*, 2002) and HPV15, 20 and 24 (Feltkamp *et al.*, 2003).

L1 of HPV11 and HPV16 expressed as fusion proteins with glutathione S-transferase (GST) in *E. coli* spontaneously form homogeneous capsomeres (Chen *et al.*, 2000, 2001; Li *et al.*, 1997). Capsomeres generated from bacterially expressed L1 of HPV11 and CoPV display neutralizing, linear and conformational epitopes and induce neutralizing antibodies upon experimental immunization (Rose *et al.*, 1998; Yuan *et al.*, 2001).

Recently, GST-HPV L1 fusion protein-based antibody detection systems have been developed for many HPV types (Karagas *et al.*, 2006; Sehr *et al.*, 2002; Waterboer *et al.*, 2005).

Monoclonal antibodies (mAb) have been generated against VLP of different HPV including types 6, 11, 16, 18, 31, 33, 35 and 45 (Christensen *et al.*, 1996a, b; Combata *et al.*, 2002; Ludmerer *et al.*, 2000). They are used to define HPV capsid epitopes.

Epitopes on HPV capsids can be experimentally classified as conformational (present on intact VLP and capsomeres) or linear (displayed by synthetic peptides and denatured L1) (Christensen *et al.*, 1996a, b). Neutralizing epitopes have also been identified in the L1 major capsid protein (Christensen *et al.*, 1994b; Giroglou *et al.*, 2001; Rose *et al.*, 1998; White *et al.*, 1998, 1999). To analyse HPV neutralization properties, many systems have been established using athymic mouse xenograft (Kreider *et al.*, 1987), raft culture systems (Meyers & Laimins, 1994) or production of infectious HPV pseudovirions *in vitro*

(Roden *et al.*, 1996; Touze & Coursaget, 1998; Unckell *et al.*, 1997). Pastrana and colleagues (Buck *et al.*, 2005; Pastrana *et al.*, 2004) have developed an *in vitro* neutralization assay utilizing HPV pseudovirions carrying a secreted alkaline phosphatase (SEAP) reporter plasmid.

This study aimed, first, to evaluate GST-L1 fusion proteins as ELISA antigens for detecting capsid-specific antibodies against alpha papillomaviruses, and second, to use GST-L1 proteins of 15 different alpha HPV types to determine type specificity and cross-reactivity of 92 mAb recognizing HPV capsid epitopes. We show that GST-L1 fusion proteins display almost all epitopes previously defined on VLP, including conformational, linear and neutralizing epitopes. Additionally, we describe a series of intra- and/or inter-species cross-reactive epitopes and show that cross-reactivity only loosely follows phylogenetic relationships. We also show that neutralizing epitopes are always conformational and mostly, but not always, type-specific.

## METHODS

**Cloning and expression of recombinant HPV L1 proteins.** The generation of GST-L1 fusion proteins from HPV6b, 16 and 18 (all three lacking 10 aa at the N terminus) has been previously described (Sehr *et al.*, 2002). Similarly, the complete L1 open reading frames (ORF) of HPV2, 3, 10, 11, 31, 32, 33, 35, 45, 52, 57 and 58 were expressed as double fusion proteins with N-terminal GST and a C-terminal undecapeptide (tag) of the SV40 large T-antigen. These constructs and characterization of the fusion proteins will be described in detail elsewhere (manuscript in preparation).

Transformation of the modified pGEX plasmids into *E. coli* strain BL21 Rosetta DE3 (Invitrogen) and further expression and treatment of the fusion proteins was performed as previously described (Sehr *et al.*, 2002).

**Anti-HPV VLP monoclonal antibodies.** Altogether, 89 tissue culture supernatants and two ascites preparations of 91 mAb were analysed (Tables 1, 2). Yet unpublished mAb were generated and characterized as previously described (Christensen *et al.*, 1990, 1994a, b, 1996a, b; Muller *et al.*, 1997; Sehr *et al.*, 2002). Seventy-three mAb were raised against VLP of nine different HPV types (HPV6, 16, 11, 18, 45, 31, 33, 52 and 35). Six mAb (the H263 series) were generated against a hybrid containing residues 1–168 of HPV11 and residues 172–505 of HPV16. Another 12 mAb were generated against HPV11 VLP containing a G131S substitution (Tables 1, 2). All antibody preparations used had an IgG concentration of at least 800 ng ml<sup>-1</sup> (Easy-Titer IgG kit; Pierce).

**Detection of linear epitopes.** Detection of epitope type was done by an immunodot blot method. Bacterial lysates containing over-expressed GST-L1 were diluted to 1 µg ml<sup>-1</sup> total lysate protein either in PBS (native conformation) or in 0.2 M Na<sub>2</sub>CO<sub>3</sub>, pH 10, 1 mM DTT (denaturing conditions) and incubated at room temperature for 30 min. For each mAb a nitrocellulose membrane was prepared with two µl aliquots each of native and denatured lysates. After drying, membranes were blocked (room temperature, 1 h) in 10% milk-PBS buffer, incubated (room temperature, 1 h) with the mAb preparations diluted 1:50 in blocking buffer, washed (6 × 10 min) in PBS and incubated (room temperature, 1 h) with peroxidase-conjugated goat anti-mouse antibody (Dianova). After washing, antibody reactions were detected using Amersham ECL Plus Western Blotting Detection System (GE Healthcare). The epitope type

of four antibody preparations that reacted neither with native nor with denatured GST-L1 could not be determined.

**GST-L1 capture ELISA.** Ninety-six-well PolySorp plates (Nunc) were coated overnight at 4 °C with glutathione-casein and blocked the next day as previously described (Sehr *et al.*, 2001). The plates were incubated (room temperature, 1 h) with the cleared GST-L1 fusion protein lysates diluted in casein blocking buffer to 0.25 µg µl<sup>-1</sup> total protein (saturating concentration) and washed. As a negative control, GST-tag lysate was used in parallel. For titrations (each mAb against the immunogen HPV L1), plates were incubated (room temperature, 1 h) with threefold dilutions (starting concentration 1:10) of the mAb in casein blocking buffer (Sehr *et al.*, 2001). For cross-reactivity analyses, mAb were incubated (room temperature, 1 h) with the 15 GST-L1. Plates were washed, incubated (room temperature, 1 h) with biotin-conjugated goat anti-mouse Ig, incubated (room temperature, 30 min) with streptavidin-poly-HRP (horseradish peroxidase), washed again and the colour reaction was developed as previously described (Sehr *et al.*, 2001). For 28 HPV16 mAb analysed at two separate days, the coefficient of determination of the linear regression of the absorption values was  $R^2=0.78$ .

**HPV16 VLP-capture ELISA.** HPV16 VLP-capture ELISA was performed as previously described (Muller *et al.*, 1997). Briefly, plates (Nunc) were coated overnight at 4 °C with rabbit anti-HPV16 VLP polyclonal antibody diluted 1:200 in PBS and blocked the next day with 5% milk, PBS, 0.05% Tween 20 (blocking buffer). As antigen, HPV16 VLP (3.5 µg ml<sup>-1</sup> in blocking buffer) generated from the same parental plasmid as GST 16L1 and purified by two gradient centrifugations as previously described (Muller *et al.*, 1997) were used. Further assay procedures were as described above for GST-L1 capture ELISA.

**Neutralization assay.** *In vitro* neutralization assays based on HPV pseudovirions carrying a SEAP reporter gene were done as previously described (Buck *et al.*, 2005; Pastrana *et al.*, 2004). Briefly, 300 000 293TT cells ml<sup>-1</sup> (293 cells transfected with an expression plasmid encoding a cDNA for SV40 large T-antigen) were seeded, infected after 5 h with a mixture of serial mAb dilutions (starting concentration 1:50) with the pseudovirions of the immunogen HPV type and incubated (5 days, 37 °C). Cells supernatants were assayed for SEAP using a chemiluminescent SEAP Reporter Gene Assay (Roche Diagnostics). Pseudovirions of HPV16, 18, 45, 6 and 11 were available. For cross-neutralization analyses, all neutralizing mAb were investigated at 1:50 dilution with the HPV pseudovirions of the other types.

**Data analysis and statistics.** In all ELISA binding experiments, lysate from bacteria expressing GST-tag alone was analysed in parallel to define the reaction background. The measured absorbances (*A*) at 450 nm were expressed in milliunits (mAU). The net *A*<sub>450</sub> value of a mAb was obtained by subtracting the background reactivity from the absorbance with the GST-L1-tag fusion protein. Monoclonal antibodies were arbitrarily classified as reactive when the *A*<sub>450</sub> value at 1:90 dilution was equal to or greater than 110 mAU. The antibody titre was defined as the last dilution yielding readings equal to or greater than 110 mAU. For cross-reactivity experiments, a mAb concentration close to saturation (mean 79% of *A*<sub>max</sub> with the immunogen HPV type; SD 15%) was used. All mAb were measured in duplicate wells and the mean of the specific reactivity of the duplicate values was taken as the final readout.

In SEAP neutralization assays, net relative light units (RLU) were calculated by subtracting RLU of cells without mAb and pseudovirions (background) from RLU of cells treated with the mAb/pseudovirion mixture. The end-point neutralization titre was defined as the last dilution yielding ≥70% reduction in the SEAP activity in

comparison to the reactivity of the pseudovirions added without antibody. For cross-neutralization, all mAb were analysed in duplicate cultures.

## RESULTS

### Reactivity of VLP-specific mAb with GST-L1 of the respective HPV type

Eighty-nine (97%) of the 92 mAb investigated reacted with the GST-L1 fusion protein of the respective immunogen HPV type (Tables 1, 2). The six mAb (H263.A2, H263.C3, H263.F4, H263.G2, H263.H9 and H263.D1) generated against a hybrid VLP containing residues 1–168 of HPV11 and residues 172–505 of HPV16 were analysed with both HPV16 and HPV11 GST-L1. H263.A2 reacted only with HPV16 while H263.G2 reacted only with HPV11 (Table 1). The other four mAb reacted more strongly with HPV11 than with HPV16 (Table 2). All 12 mAb generated against VLP of HPV11 L1 mutant G131S reacted with HPV11 GST-L1.

Of the 89 reactive mAb, 82 showed *A*<sub>max</sub> values above 1500 mAU with log end-point dilution titres (the last dilution giving a signal ≥110 mAU) varying from 3.3 to 6.3 for tissue culture supernatants and up to 7.3 for ascites as antibody source (Tables 1, 2). The titration curves of these mAb showed steep slopes, i.e. *A*<sub>450</sub> values decreasing from 80 to 20% of *A*<sub>max</sub> within 10-fold dilution. For the remaining seven mAb (H45.N5, H35.Q8, H35.H9, H35.N6, H33.E12, H33.B6 and H18.A7), the *A*<sub>max</sub> values were below 1100 mAU, log titres were between 2.0 and 4.3 and the titration curves were flat, i.e. *A*<sub>450</sub> values decreasing from 80 to 20% of *A*<sub>max</sub> only within more than 100-fold dilution. Fig. 1 shows six representative examples of mAb titration curves.

Three mAb preparations, despite estimated IgG concentrations of >800 ng ml<sup>-1</sup>, were either very weakly reactive (H6.C6 and H35.O3) or non-reactive (H16.L4) with the respective GST-L1 protein (*A*<sub>450</sub> <110 mAU at 1:90 dilution) (Table 2) and were not analysed further. For H6.C6 and H16.L4, the L1 N terminus is known to be an essential part of the epitope (Christensen *et al.*, 1996b), and the HPV6 and 16 GST-L1 protein used here lacks the 10 N-terminal amino acid residues (Sehr *et al.*, 2002). The reason for the very weak reactivity of mAb H35.O3 remains unclear. However, the reactivity of the other three mAb generated against HPV35 VLP with HPV35 GST-L1 was rather weak with low *A*<sub>max</sub> and flat titration curves (Table 1, Fig. 1).

### HPV type specificity of mAb

HPV type specificity of the 89 reactive mAb was investigated with GST-L1 fusion proteins of 15 HPV types representing six alpha papillomavirus species (α1, α2, α4, α7, α9 and α10). To allow cross-reactivity analysis in similar conditions for all antibodies, we used a mAb concentration

**Table 1.** Reactivity of mono-reactive HPV antibodies

Species	Immunogen HPV type	mAb	$A_{\max}^*$ (mAU)	mAb titre ( $\log_{10}$ )			Published data			
				E†	N‡	E/N§	Ig isotype	Epitope		
								Type	Essential aa	Ref#
$\alpha 9$	16	H16.5A	2417	6.2	3.8	2.4	G2a	C+	266–297 339–365	A P
		H16.14 J	2299	5.3	2.9	2.4		C	172–505	A
		H16.3A	2283	3.8	3.5	0.3		C+	172–505	A
		H16.1A	2292	5.3	2.9	2.4	G2	C	266–297 339–365	A P
		H16.6F	2276	6.2	3.5	2.7	G2a	C+	266–297 339–365	A P
		H16.15G	2284	5.3	—			L	172–505	A
		H16.U4	1976	6.8			G2a	C+	172–505	A B
		H16.V5	1978	7.3			G2b	C+	266–297 339–365	A B
		H16.E70	1645	3.8	2.3	1.5	G2b	C+	282	A B K
		H16.4A	1942	5.8	3.5	2.3		C+	172–505	A
		H16.L4††	164	1.0			G2a	L	N terminus	A NC
	H11:16**	H263.A2	2332	5.8	2.9	2.9		C+	266–297 339–365	A
	31	H31.A6	2171	4.3			G1	C+		A B
	33	H33.J3	2372	4.8						NC
		H33.B6	1576	4.3				C‡		NC
		H33.E12	1355	4.3				C+		B
	35	H35.H9	658	2.3						NC
		H35.Q8	1166	3.8				C‡		NC
		H35.N6	340	2.9						NC
		H35.O3††	125	1.0						NC
	52	H52.C1	2095	4.8				C‡		NC
		H52.B4	1854	4.3				C‡		NC
		H52.A7	2047	4.8				C‡		NC
		H52.D11	1980	4.8				C‡		NC
$\alpha 7$	18	H18.K2	2531	6.2	—		M	C		B
		H18.J4	2326	5.8	5.6	0.2	G2a	C		B
		H18.R5	2371	4.8	—		G2b	C		B
		H18.U15	2413	4.8	—		G1	C		B
		H18.G10	2384	5.3	4.1	1.2	G2b	C		B
	45	H45.N5	429	3.8	2.9	0.9		C		G
		H45.L10	2102	4.8	3.8	1.0		C		G
		H45.C1	2188	5.3	3.2	2.1	M	C		B
$\alpha 10$	6	H6.M48	2105	3.8	2.9	0.9	G1	C+	46 53 169–178	F I
		H6.B10.5	1856	2.9			G2b	C+	46 53 169–178	B I
		H6.N8	2347	4.3	2.3	2.0	G1	C+	46 53	F I
		H6.C6††	288	1.5			G2a	L-	N terminus	A NC
	11	H11.H3	2145	5.8	4.7	1.1	G2b	C+	132 246 345–348	A D J
		H11.F1	2178	5.3	4.4	0.9	G2a	C+	131 132 246 278	H J P
		H11.B2	2249	6.2	4.7	1.5	G2b	C+	131 132 246 278	B J
		H11.A3.2	2130	4.8	2.9	1.9	G2a	C-	49–54 170–179 246 278	D I J
		IV17B6(16)	1643	5.8	3.5	2.3		C‡		MM
		IV8D3(7)	1644	5.8	3.5	2.3		C‡		MM
		IV12D9(11)	1637	4.8	3.8	1.0		C‡		MM
		IV4A1(3)	1613	4.8	2.6	2.2		C‡		MM
		IV12D3(12)	1632	5.3	2.9	2.4		C‡		MM
	11G131S‡‡	G131S11.I1	2289	5.3	4.1	1.2	G1	C+	262–289	D
		G131S11.G3	2396	5.8	4.7	1.1	M	C+	262–289 345–348	D
		G131S11.C2	2121	5.3	2.9	2.4	G1	C+		D
	H11:16	H263.G2	1685	3.8	2.9	0.9		C‡	1–168	A

\* $A_{450}$  at 1:10 dilution in ELISA with GST–L1 of immunogen HPV type; mAU, absorbance milliunits.

†ELISA titre.

‡Neutralization titre.



**Table 1.** cont.

§Log<sub>10</sub> of ratio of ELISA to neutralization titre.

||Reported epitope and neutralization type; C, conformational; L, linear; +, neutralizing; -, non neutralizing;

¶, epitope type is defined in this study.

#References: A, Christensen *et al.*, 2001; B, Christensen *et al.*, 1996a; D, Ludmerer *et al.*, 2000; F, Christensen *et al.*, 1996b; G, Combita *et al.*, 2002; H, Ludmerer *et al.*, 1997; I, McClements *et al.*, 2001; J, Ludmerer *et al.*, 1996; K, Roden *et al.*, 1997; MM, Martin Müller, unpublished; NC, Neil D. Christensen, unpublished; P, Culp *et al.*, 2007.

\*\*H11:16, hybrid containing aa 1–168 of HPV11 and 172–505 of HPV16.

††mAb not analysed further.

‡‡Mutant HPV11 with G131S substitution.

below but close to saturation for the immunogen HPV type (Fig. 1).

Under these conditions, 46 (52 %) of the mAb reacted only with the GST–L1 fusion protein of the HPV type used for immunization (Table 1), while the other 43 mAb (48 %) showed different levels of intra- and/or inter-species cross-reactivity (Table 2).

Of the 43 cross-reactive mAb, 23 (54 %) showed strong ( $A_{450}$  relative to the type used for immunization >80 %), 11 (26 %) showed intermediate (relative  $A_{450}$  50–80 %) and 9 (20 %) showed weak (relative  $A_{450}$  <50 % but absolute  $A_{450}$  >110 mAU) cross-reactivity with at least one other HPV type. Of the 153 cross-reactions, 66 (43 %) were strong, 34 (22 %) were intermediate and 53 (37 %) were weak. This suggests that the cross-reactive epitopes recognized by about half of the cross-reactive antibodies and in about half of all cross-reactions are very similar or even identical to the specific epitope.

All mAb reactive with HPV31 ( $n=1$ ), HPV35 ( $n=3$ ) and HPV52 ( $n=4$ ) were monotypic.

Of the 27 mAb generated against HPV16 VLP, 16 (59 %) were cross-reactive. Two mAb showed strictly intra-species and four showed strictly inter-species cross-reactivity. The other 10 mAb showed mixed intra/inter-species cross-reactivity. Most frequent intra-species cross-reactions were with HPV35 ( $n=10$ ), HPV31 and HPV58 (both  $n=9$ ), followed by HPV33 ( $n=7$ ). HPV52 L1 ( $n=4$ ) showed the least frequent cross-reactivity. On the other hand, the most frequent inter-species cross-reactions were with HPV18 ( $n=12$ ), followed by HPV45 ( $n=8$ ), HPV11 ( $n=7$ ) and HPV6 ( $n=6$ ). Cross-reactivity was less frequent with HPV2 and HPV32 (both  $n=4$ ), HPV10 ( $n=3$ ), and HPV57 and HPV3 (both  $n=2$ ). Five HPV16 mAb cross-reacted also with skin alpha papillomavirus types; in this group, cross-reactivity was weak or absent with low-risk mucosal HPV types 6, 11 and 32, but strong or intermediate with the high-risk HPV types 18 and 45. Antibody H16.11B, which recognized a conformational epitope, showed the broadest cross-reactivity. It reacted with all 15 alpha papillomaviruses analysed, strongly with 12 HPV types and weakly with types 6, 11 and 32.

From the four HPV33 mAb, only one cross-reacted strongly with HPV52 and HPV58 and weakly with

HPV31, all from its own  $\alpha 9$  species. In addition, a strong but isolated cross-reaction was seen with the phylogenetically distantly related HPV32.

Of the 11 HPV18 mAb, 6 (55 %) were cross-reactive. They all showed mixed intra- and inter-species cross-reactivity and reacted with the closely related HPV type 45. Five of them reacted also with skin HPV types, i.e. HPV2 and HPV57, and four also with HPV3 and HPV10. Fourteen out of the 18 cross-reactions with skin types were strong ( $n=11$ ) or intermediate. Antibody H18.A7, which reacted only weakly with HPV18, showed very broad although mostly weak ( $n=7$ ) or intermediate ( $n=6$ ) cross-reactions with all other HPV tested. The other five mAb cross-reacted with neither the mucosal high-risk HPV of the  $\alpha 9$  nor the low-risk HPV of the  $\alpha 10$  and  $\alpha 1$  species, which is in contrast to the frequent cross-reactions of HPV16 mAb with HPV18.

Of the five HPV45 mAb, two showed strong and intermediate intra-species cross-reactivity while inter-species cross-reactivity was absent.

HPV6 and 11 are very closely related low-risk types of the  $\alpha 10$  species. Of the 34 mAb raised against these two types, 18 (53 %) were cross-reactive, all recognized both HPV types. Inter-species cross-reactivity was rare with only four mAb additionally reacting weakly or intermediate with one or two types of the high-risk HPV.

### Direct comparison of mAb reactivity with HPV16 GST–L1 and VLP

In a direct experimental comparison, 32 mAb, encompassing 28 raised against HPV16 and the 4 generated against other HPV types but cross-reactive with HPV16 GST–L1, were also analysed by HPV16 VLP ELISA. All HPV16-specific mAb reacted in VLP-ELISA, including H16.L4, which was non-reactive in GST–L1 ELISA; this mAb is known to detect an epitope located within the N terminus of L1 (Christensen *et al.*, 1996a; Christensen, unpublished) that is deleted in HPV16 GST–L1. GST–L1 and VLP titres were similar (GST–L1 to VLP titre ratio: median 2.9, range 0.04–27) for the 14 neutralizing mAb, but GST–L1 titres were much higher for the 13 non-neutralizing mAb (222, 3–6000). Also the 11 monospecific mAb had similar GST–L1 and VLP titres (1, 0.05–10), whereas the GST–L1 titres

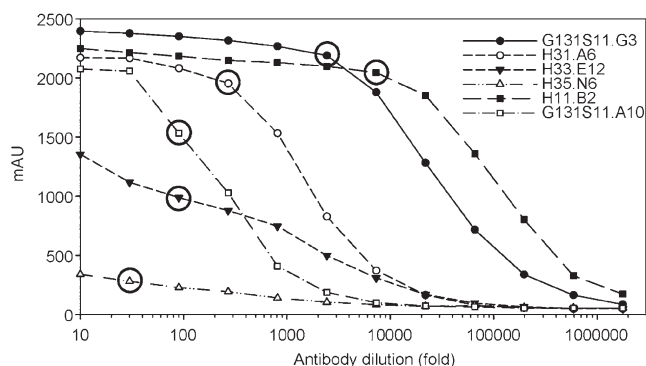
**Table 2.** Reactivity of cross-reactive HPV antibodies

Species Immunog. HPV type			A <sub>max</sub> <sup>*</sup> (mAU)	Ab Titre (log <sub>10</sub> )		ELISA Reactivity (mAU) with GST-L1 of specific HPV types															Published data																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																			
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[illegible]

###Also cross-neutralizes HPV11 with  $\log_{10}$  of 2.6.



**Fig. 1.** Representative dose-response curves for binding of HPV VLP-specific mAb to GST-L1 fusion proteins. ELISA analysis of serial dilutions of mAb preparations incubated with GST-L1 fusion proteins immobilized via glutathione-casein to the ELISA plates. Each mAb was analysed with the L1 protein of the immunogen HPV type. Circles indicate the dilution used for cross-reactivity analyses.

of the 16 cross-reactive mAb were always higher (50, 2.5–6500). Finally, the GST-L1 to VLP titre ratios were higher for the 9 mAb recognizing linear (27, 8–6000) than for those 9 mAb recognizing conformational epitopes (3, 0.04–800). In summary, GST-L1 display neutralizing, mono-specific and conformational epitopes like VLP, but GST-L1 appears to display non-neutralizing, cross-reactive and linear epitopes in larger quantity than highly purified, antibody-captured VLP.

### Neutralization activity of VLP-specific mAb

Seventy four mAb were analysed for neutralization of HPV6, 11, 16, 18 and 45 (Tables 1, 2). Thirty four (46%) mAb neutralized pseudovirions of the immunogen HPV type. End-point neutralization log titres ranged from 1.7 to 5.6. Thirty three of the neutralizing mAb recognized a conformational epitope and one (MM07) recognized a linear epitope. Among mAb recognizing monospecific epitopes, 28 out of 42 (67%) were neutralizing, in contrast to only five out of 29 (17%) mAb recognizing cross-reactive epitopes.

All neutralizing mAb were further investigated at a 1:50 dilution for cross-neutralization with pseudovirion preparations of HPV6 and 11 ( $\alpha$ 10), 16 ( $\alpha$ 9), as well as 18 and 45 ( $\alpha$ 7). Cross-neutralization was observed only once; mAb H6.L12 neutralized both HPV6 and the most closely related HPV11 pseudovirions.

## DISCUSSION

This study represents the largest and most comprehensive analysis conducted so far on presence and type specificity of epitopes displayed by HPV capsid proteins. It introduced 25 new mAb and further characterized 24 previously

published mAb. It reproduced published data for 36 out of 42 mAb and the only differences observed were the extremely weak binding reactivity of H35.O3 and H6.C6, and the absence of binding (H16.L4) or neutralization activity of mAb H16.J4 with HPV16, and G131S11.H5, -.D2 and -.A10 with HPV11.

The majority of published HPV serology data has been established using VLP-based ELISA assays. Here, we used HPV L1 antibody assays based on bacterially expressed, affinity-purified glutathione *S*-transferase L1 (GST-L1) fusion proteins as antigens (Sehr *et al.*, 2002). This assay has been previously validated for HPV16 and 18 with human and experimental mouse sera by comparison with VLP-ELISA data (Davidson *et al.*, 2003; Sehr *et al.*, 2002). The bacterial expression system allowed analysing 9 mucosal HPV types that had been available before as VLP and 6 HPV types, i.e. skin HPV2, 3, 10 and 57 as well as the mucosal high-risk HPV type 58 and low-risk HPV type 32 that had not been available before as VLP. These numbers reflect the ease with which GST-L1 fusion proteins can be generated, purified and applied in antibody assays.

The binding of 89 (97%) of the 92 mAb indicates that GST-L1 fusion proteins (with the exception of HPV35) present most (if not all) of the antigenic epitopes presented by VLP. Only two mAb reacted extremely weakly and one did not react with the GST-L1 protein of the respective HPV type. H6.C6 and H16.L4 detect an epitope located on the N terminus of L1 (Christensen *et al.*, 1996a, b), and GST-L1 preparations of HPV6 and 16 lack aa 1–10. Fusion of GST to the L1 N terminus apparently did not inhibit binding of any other mAb, indicating that all of them are either targeting epitopes elsewhere or that GST fusion does not affect N-terminal epitope conformation (Sehr *et al.*, 2002).

Four HPV35 specific mAb reacted very weakly, suggesting that the HPV35 GST-L1 preparation lacked most of the VLP-displayed epitopes for yet unknown reasons. However, the presence of 3 conformational cross-reactive epitopes (H16.8B, H16.11B and H263.C3) indicates at least partially correct conformation of HPV35 GST-L1. H18.R5, reported to recognize a conformational epitope on HPV18 and HPV45 VLP, reacted only with HPV18 GST-L1, suggesting a subtle epitope difference of HPV45 GST L1.

Our results show that GST-L1 fusion proteins have the epitope repertoire of intact VLP (conformational), but also display epitopes presented by denatured VLP (linear). Linear epitopes are also present on intact VLP preparations either because they are surface-exposed on capsids or because the VLP preparation also contains some denatured L1, but reactions with intact VLP are usually weaker than with denatured proteins (Christensen *et al.*, 1996a). Our direct comparison using twofold gradient-purified and antibody-captured HPV16 L1 VLP as ELISA targets also demonstrated the presence of all linear and all cross-reactive epitopes in VLP ELISA, but in clearly lower density than in GST-L1 ELISA.

Of the 89 mAb reacting with GST-L1, 71 are known to recognize conformational epitopes, including 34 neutralizing epitopes, and 14 are known to recognize linear epitopes, of which 8 were reported also to react with some intact VLP preparations (see Tables 1 and 2 for mAb and references). These findings suggest that the GST-L1 fusion protein preparations display neutralizing and conformational as well as linear epitopes. Two mAb recognizing linear epitopes (H16.B20, H16.S1) did not react with GST-L1 of HPV33 or 31, respectively, in contrast to their reported cross-reactivity with denatured VLP of these types, indicating that the GST-L1 preparations of these types presented these linear epitopes in insufficient amounts.

The titres of the different mAb preparations used in this study varied by several orders of magnitude. Choosing of the first dilution below saturation for cross-reactivity experiments allowed comparing the strength of the cross-reactivity signals and classifying them into three categories, strong, intermediate or weak. In a previous, smaller analysis of VLP cross-reactivity of some of the mAb used here, only a uniform dilution of 1:50 of tissue culture supernatants irrespective of the effective antibody concentration had been applied (Christensen *et al.*, 1996a).

Among the 89 reactive mAb, 46 reacted only with GST-L1 proteins of the specific HPV type used for immunization, indicating that GST-L1 fusion proteins are displaying type-specific epitopes. Although we analysed cross-reactivity with 15 alpha papillomavirus types, as compared to a maximum of 9 types in previous VLP studies, we cannot exclude that some of the mAb classified here as monotypic might turn out to be cross-reactive when analysed with additional types. Previously published data suggested that conformation-dependent mAb H16.11B and H16.8B might be specific for HPV16 alone (Christensen *et al.*, 2001), but here we describe strong cross-reactivity to many other

mucosal and skin alpha papillomaviruses. In contrast, the cross-reactivity to HPV11 GST-L1, which was not seen with HPV11 VLP, was very weak. For mAb H16.7E we confirm absence of cross-reactivity to HPV11 (Christensen *et al.*, 2001), but observed a weak cross-reactivity to HPV35. However, we did not see any reactivity to the four other HPV types in the same species ( $\alpha 9$ ). For mAb H16.2F we confirm the absence of cross-reactivity to HPV11 (Christensen *et al.*, 2001), but we additionally found weak cross-reactivity with HPV18 and HPV45 (both  $\alpha 7$ ).

We describe new cross-reactivity for 12 additional antibodies, including 5 raised against HPV16, one against HPV18, two against HPV45, one against HPV33 and three against the HPV11 G131S L1 mutant. Moreover, we extend the cross-reactivity pattern for 12 mAb. Altogether we have data for 43 cross-reactive monoclonal antibodies, encompassing a total of 153 individual cross-reactions. This provides a comprehensive description of some features of cross-reactive epitopes among human papillomaviruses.

Cross-reactivity follows only loosely the phylogenetic grouping. Among very closely related HPV types, i.e. HPV6b and 11 (>92 % aa identity in L1) as well as 18 and 45 (88 %), cross-reactivity is frequent (Table 3). Since no other HPV types from species  $\alpha 10$  and  $\alpha 7$  were analysed, it is unclear whether there might be more intra-species cross-reactivity among these antibodies. Only two of the 17 cross-reactive mAb raised against a HPV type of the  $\alpha 9$  species were strictly intra-species cross-reactive. These two mAb both recognized only a subset of the six HPV types analysed from this species, suggesting that species-specific epitopes are rare or even do not exist. Furthermore, most mAb with inter-species cross-reactivity did not react with all members of the species of the HPV type against which they were raised, indicating again that HPV species may not share common epitopes.

**Table 3.** Summary of mAb reactivities

Immunogen* HPV type	Reactivity			Cross-Reactivity Pattern			
	All	Mono-	Cross-	All	Intra-species	Inter-species	Mixed
All	89	46	43	43	18	4	21
$\alpha 9$							
16	27	11	16	16	2	4	10
31	1	1					
33	4	3	1	1			1
35	3	3					
52	4	4					
$\alpha 7$							
18	11	5	6	6			6
45	5	3	2	2	2		
$\alpha 10$							
6	8	3	5	5	4		1
11	26	13	13	13	10		3

\*For grouping of mAb against hybrid H11:16 and mutant 11G131S see Tables 1 and 2.

There were no mAb specific for all mucosal alpha papillomaviruses analysed that also lacked reactivity to any of the 4 alpha skin HPV. Many other cross-reactive mAb did not recognize some of the most closely related HPV, but rather showed cross-reactions with individual, distantly related HPV of other species, including alpha skin HPV described here for the first time. Two of the HPV16 mAb (H16.H5 and H16.9A) did not react with any of the 5 other HPV in species  $\alpha 9$  but instead reacted selectively with HPV18 L1. The weak cross-reaction suggests that the epitope recognized on HPV18 is not identical to that on HPV16. Antibody H16.H5 has been described to bind HPV16 L1 peptide aa 174–185 (Christensen *et al.*, 1996a). HPV18 has 7 mismatches in the homologous sequence, suggesting that weak cross-reactivity is not mediated by this sequence. On the other hand, two other HPV16 mAb (Ritti01 and MM07) reacted with 5 of the 6  $\alpha 9$  HPV types and very selectively also with HPV45. One of them reacted marginally even with skin HPV2 but not with closely related skin HPV57. Monoclonal antibody H16.S1 cross-reacted with mucosal HPV types including HPV35 ( $\alpha 9$ ), HPV18 and 45 ( $\alpha 7$ ) and additionally with skin HPV types 3 and 10 ( $\alpha 2$ ). It has been described to bind HPV16 L1 peptide aa 111–130, (Christensen *et al.*, 1996a). Leucine residues 122 and 126 appear to be critical since all non-reactive HPV types have replaced at least one of them by an aromatic residue and the weakly cross-reactive HPV10 differs at the neighbouring position (D127E).

While HPV18 was the type most frequently recognized by cross-reactive HPV16 mAb (12 out of 16), only one (pan-reactive H18.A7) of the 6 cross-reactive HPV18 mAb recognized HPV16. Most frequently observed inter-species cross-reactivity was directed against skin HPV3, 10 and/or 2, 57. Antibody H18.Q2 cross-reacted with HPV types 45, 2 and 57.

Of 14 mAb detecting a linear epitope, 13 were inter-species cross-reactive and one (H16.15G) monotypic (Table 4). Of 71 mAb detecting conformational epitopes, 29 (41 %) were cross-reactive. While 16 of these recognized only closely related HPV types (6/11 and 18/45), three showed weak and four strong inter-species cross-reactions even including skin HPV types. In conclusion, the cross-reactivity observed here suggests that distantly related mucosal and skin alpha HPV share conformational epitopes and that the

phylogenetic L1-based species definition may not define a serological unit since no species-specific epitope was found.

For H16.J4, neutralizing activity was described by Combata *et al.* (2002), whereas in our experiments this antibody showed no HPV16 neutralization.

For five of the six cross-reactive neutralizing mAb, cross-neutralization could be assessed. H6.L12 was strongly cross-reactive with the very closely related HPV11 and neutralized HPV11 with a similar titre as HPV6, which is in agreement with previously published data (Christensen *et al.*, 1996b). Antibodies H16.2F, H6.F62 and Ritti01 and MM07 were weakly cross-reactive with HPV18/45, HPV11, and HPV45 (Ritti01 and MM07), respectively, but showed no cross-neutralization with these types. The weak cross-reaction indicates lower affinity of the mAb to the cross-reactive epitope, which might be insufficient to induce neutralization on the cross-reactive epitope, alternatively the absence of cross-neutralization might indicate that the cross-reactive epitope is functionally different, i.e. that antibody binding does not lead to neutralization.

GST–L1 ELISA titres were always higher than neutralization titres, as has been described previously (Combata *et al.*, 2002; White *et al.*, 1999) (Tables 1, 2). ELISA log titres on average were similar among monospecific (median 5.3, range 3.8–6.2) and cross-reactive mAb (5.0, 2.0–6.2). However, cross-reactive mAb rather consistently showed lower neutralization log titres (2.5, 1.7–3.8) than the monospecific mAb (3.5, 2.3–5.6). Thus, the ratio of ELISA to neutralization titres was on average about 100-fold higher among the cross-reactive (log median 3.3, range 2.4–3.6) than among the monospecific mAb (1.5, 0.2–2.9). This may indicate that neutralization epitopes are highly type-specific. The cross-reactive antibodies that are monospecific in neutralization might recognize the L1 surface involved in neutralization (functional structure) only partially because they also recognize neighbouring, non-functional structures shared by other types, thus their ability to induce a structural change or to block a surface essential for the infection process is reduced, but could be overcome in higher concentrations, resulting in lower neutralization titres.

In conclusion, HPV capsid epitopes defining neutralizing sites are always conformational and most of the mAb

**Table 4.** Association of cross-reactivity of HPV mAb with linear and conformational epitopes

Epitope	Reactivity				Neutralizing activity
	All	Monotypic	Cross-reactive		
			Intra-species only	Inter-species or mixed	
Total	85	43	16	26	34
Conformational	71	42	16	13	33
Linear	14	1	0	13	1



detecting monospecific conformational epitopes are neutralizing. Monoclonal antibodies binding to conformational cross-reactive epitopes are rarely neutralizing, and if neutralizing they are rarely cross-neutralizing. Monospecific mAb neutralize more efficiently than cross-reactive.

In summary, our data indicate that bacterially expressed GST-L1 fusion proteins display the same epitopes as VLP and therefore fulfil the essential requirements of antigens for HPV serology. They are useful tools to define and to recognize complex patterns of conformational and linear cross-reactive epitopes. However, when extrapolating these data to the analysis of human sera, it should be kept in mind that the experimentally produced mAb analysed here and human antibodies generated by natural HPV infection may differ due to differences in kind of antigen (recombinantly produced and purified VLP versus native virions), host producing the antibodies (mouse versus human), and site, dose and kinetics of immunization (long-term and low dose in natural infection versus short-term boosting with high doses in experimental animals).

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## **CHAPTER THREE**



## **2<sup>nd</sup> Contribution**

### **Absence of SV40 antibodies or DNA fragments in pre-diagnostic mesothelioma serum samples**

Kjærheim *et al.*, *Int J Cancer*, **120**, 2459-2465.

## Absence of SV40 antibodies or DNA fragments in prediagnostic mesothelioma serum samples

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The rhesus monkey virus Simian Virus 40 (SV40) is a member of the polyomavirus family. It was introduced inadvertently to human populations through contaminated polio vaccine during the years 1956–1963, can induce experimental tumors in animals and transform human cells in culture. SV40 DNA has been identified in mesothelioma and other human tumors in some but not all studies. We tested prediagnostic sera from 49 mesothelioma cases and 147 matched controls for antibodies against the viral capsid protein VP1 and the large T antigen of SV40 and of the closely related human polyomaviruses BK and JC, and for SV40 DNA. Cases and controls were identified among donors to the Janus Serum Bank, which was linked to the Cancer Registry of Norway. Antibodies were analyzed by recently developed multiplex serology based on recombinantly expressed fusions of glutathione-S transferase with viral proteins as antigens combined with fluorescent bead technology. BKV and JCV specific antibodies cross-reactive with SV40 were preabsorbed with the respective VP1 proteins. Sera showing SV40 reactivity after preabsorption with BKV and JCV VP1 were further analyzed in SV40 neutralization assays. SV40 DNA was analyzed by SV40 specific polymerase chain reactions. The odds ratio for being a case when tested positive for SV40 VP1 in the antibody capture assay was 1.5 (95% CI 0.6–3.7) and 2.0 (95% CI 0.6–7.0) when only strongly reactive sera were counted as positive. Although some sera could neutralize SV40, preabsorption with BKV and JCV VP1 showed for all such sera that this neutralizing activity was due to cross-reacting antibodies and did not represent truly SV40-specific antibodies. No viral DNA was found in the sera. No significant association between SV40 antibody response in prediagnostic sera and risk of mesothelioma was seen.

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**Key words:** polio vaccination; viral infection; simian virus 40; malignant mesothelioma

The rhesus monkey virus Simian Virus 40 (SV40), introduced inadvertently to human populations through contaminated polio vaccine during the years 1956–1963, has repeatedly been shown to induce tumors in animal models and to transform human cells in culture (reviewed in Refs. 1,2). Many types of tumors have been observed, including mesothelioma, ependymoma, osteosarcoma and non-Hodgkin lymphoma, apparently partly dependent on the route of infection.<sup>2</sup>

The strong and consistent relation between experimental SV40 infection and cancer development in rodents has motivated the investigation of its carcinogenic potential in humans. SV40 DNA sequences have repeatedly been reported in cases of human cancers of essentially the same types as has been seen in rodents.<sup>3–7</sup> In a meta-analysis including 528 mesothelioma cases and 468 controls from 15 studies, the combined OR for the presence of SV40 DNA sequences in mesothelioma tumor tissue was 17 (95% CI

10–28).<sup>8</sup> SV40 early region sequences have also been detected in human peripheral blood cells, and in preparations of B and T lymphocytes from healthy subjects.<sup>9</sup> Other studies of SV40 sequences in tumor tissue have however seriously questioned these results, suggesting positive findings to be caused by contamination or other laboratory artifacts.<sup>10–12</sup>

Available epidemiological studies have mostly been based on proxies of infection, such as being part of a population likely to have been exposed to contaminated vaccine.<sup>13</sup> The proportion of actually vaccinated persons and the proportion of actually contaminated vaccines used are unknown factors. Descriptive studies from the United States, Denmark, Sweden and Norway have not shown higher cancer incidence among individuals likely to have been exposed to contaminated vaccines.<sup>14–17</sup> A limitation of these studies is that the cohorts assumed unexposed to SV40 are born after 1963, and therefore in general too young to be at risk of most cancer types. A case-control study from the United States where individual vaccine status was ascertained did not support an association between SV40 and the development of brain tumor, mesothelioma or non-Hodgkin lymphoma.<sup>18</sup>

Knowledge of the extent and the natural course of SV40 infection among humans is still relatively scarce. In groups assumed to have been exposed to contaminated vaccine 12–24% were seropositive for neutralizing antibodies, while in groups born after vaccines were SV40-free seropositivity rates of 2–13% were seen.<sup>19–21</sup> Among laboratory workers handling monkeys or monkey kidney cells 40–55% have been found to be positive for SV40 neutralizing antibodies,<sup>20,22</sup> and in zoo workers with direct contact with nonhuman primates 25% had a positive response when an enzyme immunoassay was used.<sup>23</sup> Infection with human polyomaviruses BK (BKV) and JC (JCV), which are closely related to SV40, is frequent and leads to strong antibody responses that can cross-react with SV40 antigens.<sup>24</sup> After performing competitive inhibition experiments using BKV and JCV, the prevalence of seropositivity in zoo workers was reduced to 10%.<sup>23</sup> Results of old and recent studies are thus difficult to compare because of use of different assay principles and varying sensitivities. Serological studies published so far on antibodies to SV40 among cancer

**Abbreviations:** BKV, BK virus; CI, confidence interval; GST, glutathione-S transferase; JCV, JC virus; MBP, maltose binding protein; MFI, median fluorescence intensity; OR, odds ratio; SV40, simian virus 40; Tag, Large T antigen; VP1, Viral capsid protein 1.

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patients and their controls have indicated low prevalence of antibodies to SV40,<sup>25</sup> and a lack of association between cancer risk and the presence of antibodies to viral proteins when measured both at the time of diagnosis<sup>26,27</sup> and prior to diagnosis.<sup>28</sup>

The interpretation of the repeated finding of SV40 in human tumors is controversial. The question is whether SV40, if present, is a causal factor in carcinogenesis, a cofactor acting only in concert with other carcinogens (*e.g.* asbestos, in the case of mesothelioma), an expression of the reactivation of a latent infection in an immunocompromised host, or simply a result of laboratory contamination.<sup>11</sup> Under the assumption that SV40, like BKV and JCV, infects and replicates in humans and persists in a latent infection, an antibody response similar in type and extent to that against BKV and JCV should be expected. Thus, SV40-specific antibodies would be detectable in the phase before cancer development. A difference in infectious activity of SV40 measured by elevated antibodies in subjects that eventually developed mesothelioma *versus* controls could indicate an association of SV40 and human cancer. Viraemia, with identifiable viral DNA sequences in serum, may be expected to be present during the acute infection, but previous exposure may not necessarily be reflected by the presence of SV40 DNA at a later point in time.

The aim of the present study was to investigate, for the first time, the risk of mesothelioma associated with the presence of SV40 antibodies and SV40 genomic DNA in a study based on pre-diagnostic serum samples from mesothelioma cases and controls. In addition, the sera were investigated for the presence of antibodies to the human polyomaviruses BK and JC. The 3 viruses have about 70% genomic similarity with each other.<sup>29</sup> The sera were analyzed for antibodies to the major capsid protein VP1 (VP1) and to the large T antigen (Tag), identified as the oncoprotein that binds and inactivates p53 and pRb, cellular proteins with tumor suppressor activity.<sup>30</sup> To evaluate the SV40 VP1-reactive sera in the presence and absence of SV40 cross-reactive BKV and JCV specific antibodies all antibody measurements were performed with and without preabsorption of the sera with an excess of soluble VP1 proteins from BKV and JCV. Those sera that still reacted positively with SV40 VP1 when preabsorbed with BKV and JCV VP1 were further characterized in SV40 neutralization assays.

## Material and methods

### *On the vaccination program in Norway*

In Norway an estimated 3–5 million doses of potentially contaminated vaccine were used during 1956 through 1962. The total population at the time was approximately 3.5 millions. Through the whole period the Salk inactivated vaccine was used. The Sabin live, attenuated oral preparation was not given until 1965, when vaccines were free from contamination. The national vaccination program varied somewhat during the period, but in general prescribed 3 doses for children during their first year and 1 dose at age seven. In addition, vaccination was recommended for pregnant women and persons aged 18 and below. Accordingly, 3 levels of risk of SV40 infection through vaccination can be discerned; those born 1921–1937 were possibly vaccinated, those born 1938–1948 were probably vaccinated, and those born 1949–1962 were almost certainly vaccinated with potentially SV40-contaminated vaccine. No specific information on the proportion of contaminated vaccine or the level of contamination of the vaccines used in Norway was available. A more detailed description of the Norwegian polio vaccination program has been published previously.<sup>17</sup>

### *Identification of cases and selection of controls*

The study base was defined as the cohort comprised by the Janus Serum Bank, established in 1972 and presently containing more than 600,000 serum samples from over 300,000 healthy individuals. Samples have been collected in conjunction with repeated regional health surveys and from blood donors. Samples are stored at –25°C. The serum bank has previously been used in other

studies of viral markers.<sup>31–33</sup> The cohort was linked to the Cancer Registry of Norway to identify all cases with a mesothelioma diagnosis. Since 1953 all new cancer cases in Norway have been recorded at the cancer registry based on the compulsory reporting from all clinical and histopathological departments in the country. The registry is considered to be virtually complete, especially for solid tumors.

Between 1973 and 2003 altogether 1,251 new cases of mesothelioma were reported to the Cancer Registry. Of these, 82 cases (6.6%) had donated serum to the serum bank prior to their diagnosis, and were eligible for inclusion in the study. Basis for selection was the classification of the case as a malignant mesothelioma (codes 9050/3–9053/3 according to the 2nd edition of the International Classification of Oncology<sup>34</sup>). For 2 cases there was not enough serum available for the analyses, and these cases had to be excluded. To revise the histological diagnoses archival histopathological tissue samples were collected and examined with at least 4 immunohistochemical stainings<sup>35,36</sup> and reviewed independently by 3 pathologists in several steps. Eleven of the selected cases were diagnosed as adenocarcinomas or other rare tumors of the pleura or peritoneum, and in 20 cases no revision could be made because of lack of representative material. This left 49 cases to be included in the study.

For each mesothelioma case 3 controls free from cancer at the time when the case was diagnosed, matched on age ( $\pm 1$  year), gender, period of blood sampling ( $\pm 6$  months) and county, were selected from the serum bank. A total of 147 controls were thus included in the study.

### *Information on asbestos exposure*

Asbestos exposure is the main risk factor for mesothelioma,<sup>37</sup> the estimated etiological fraction for men in Norway being 84%.<sup>38</sup> Information on occupation in 1960, 1970 and 1980 was available from national censuses, coded according to The Nordic Classification of Occupations.<sup>39</sup> All occupations in the 1970 census have previously been classified as having high, moderate or little/no asbestos exposure.<sup>40</sup> The same classification was here used for the 1960 and 1980 censuses. Indicators of ever *vs.* never exposure to asbestos in the 3 censuses were combined to adjust for asbestos exposure at 4 levels (never exposed, or highly or moderately exposed at one, 2 or 3 censuses, respectively).

### *Serological analyses: Antibody capture assays*

Serological analyses to measure antibodies to the chosen series of viral proteins were conducted at the German Cancer Research Center in Heidelberg, Germany. Frozen serum samples were shipped on dry ice. They were analyzed for antibodies to VP1 and to Tag of BKV and JCV and SV40. The antibody detection method was based on glutathione-S-transferase (GST) capture ELISA as described by Sehr *et al.*<sup>41,42</sup> in combination with fluorescent bead technology as recently described.<sup>43</sup>

Briefly, full-length viral proteins were expressed in bacteria in fusion with an N-terminal GST domain. Glutathione cross-linked to casein was coupled to fluorescence-labeled polystyrene beads (MultiAnalyte, Luminex, Austin, TX) and GST fusion proteins were affinity-purified on the beads directly in a one-step procedure. Bead types of different color and each carrying a different antigen were mixed and incubated with human sera at 1:100 dilution. Antibody bound to the beads *via* the viral antigens was stained by biotinylated anti-human-Ig and streptavidin-R-phycoerythrin. Beads were examined in a Luminex 100 analyzer (Luminex) that identifies the bead color—and thus the antigen carried by the bead—and quantifies the antibody bound to viral antigen *via* the median R-phycoerythrin fluorescence intensity (MFI) of at least 100 beads of the same internal color.

Background MFI (reactivity with GST alone, usually below 100 MFI) was subtracted from the MFI values obtained with specific proteins to obtain net MFI. Negative values were set to 0. Cut-off points of 175 and 650 MFI were used for all VP1 and T-antigen

proteins, respectively. This was defined arbitrarily before the code of case-control status was broken based on visual inspection of the cumulative histograms of the distributions of viral antibodies to correspond with a common level where the MFI values started to rise. Positive and negative standard sera were included in all analyses. High sensitivity and specificity of SV40 VP1 and Tag antibody detection by these antigens was found in extensive analyses using serum panels from SV40-infected and uninfected rhesus monkeys and comparison with neutralization and immunofluorescence assays (Sehr P, Viscidi R, Shah K, Pawlita M. Detection of antibodies to VP1 and large T-antigen of polyomaviruses BK, JC and SV40. Manuscript in preparation).

Specific blocking proteins in the serum dilution buffer were used to evaluate the specificity of the SV40 VP1 antibodies. For this purpose the VP1 proteins of the polyomaviruses and the L1 protein of Human papillomavirus type 16 were bacterially expressed in full length as fusion proteins with an N-terminal maltose binding protein (MBP). To analyze polyomavirus antibodies without specific blocking, sera were preincubated with MBP-HPV16 L1 lysate (2 mg/ml total lysate protein). To block BKV and JCV antibodies that could cross-react with SV40 VP1, lysates with MBP-VP1 proteins of BKV and JCV (1 mg/ml total lysate protein each) were used simultaneously (BKV/JCV VP1 block). For further specificity analysis lysate (1 mg/ml total lysate protein) with MBP-VP1 of SV40 or of the B-lymphotropic polyomavirus (LPV), another primate polyomavirus that may infect the human population<sup>44</sup> was added to the BKV/JCV VP1 block.

#### *Serological analyses: SV40 neutralization assays*

For neutralization assays immortal African green monkey kidney cells (CV-1) were seeded on 48-well plates at a density of 6,000 cells per well and grown for 8 hr at 37°C in a 5% CO<sub>2</sub> atmosphere. Sera were diluted 1:50 in cell culture medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum) and incubated with SV40 virions for 30 min on ice. Cells were infected with the mixture and grown overnight, before the cell culture medium was changed. After 48 hr of infection, cells were harvested and seeded in duplicates on 15-well adhesion slides (BioRad, München, Germany) at a density of 3,000 cells per well. After 6 hr of incubation, the cells were fixed overnight with acetone/methanol (1:1) at -20°C. On the next day, SV40 T-antigen positive cells were stained with an experimental hamster anti-SV40 tumor serum (1:250 dilution) and Cy2-labeled goat anti-hamster Ig serum (Dianova, Hamburg, Germany; 1:500 dilution). Nuclei were counterstained with DAPI at 1 µg/ml. SV40 infection led to >50% immunofluorescence positive cells within 48 hr of infection. As a positive neutralization control, an experimental rabbit-anti-SV40 virion serum (1:500 dilution, SV40 VP1 titer >25,000) was used (<1% immunofluorescence positive cells). Means of duplicate values were computed, and samples with <10% immunofluorescence positive cells were considered neutralization positive.

Sera showing SV40 neutralizing activity without preabsorption were preabsorbed and reanalyzed. Serum preabsorption for neutralization assays was carried out differently from serum preabsorption for antibody capture assays, because presence of crude bacterial lysate was toxic for the cells. Sera were preabsorbed with GST fusion proteins of either HPV16 L1 or BKV and JCV VP1 or SV40 VP1 immobilized and purified on Glutathione-Sepharose. Hundred microliter Glutathione-Sepharose (GE Healthcare) per serum were incubated for 30 min at room temperature with freshly prepared lysate from bacteria expressing GST fusion proteins of HPV16 L1 and the Polyomavirus VP1 proteins. The sepharose was pelleted (500 rpm, 5 min) and washed twice with cell culture medium. Four microliter serum was mixed with 100 µl sepharose beads and 100 µl medium (initial serum dilution 1:25) and incubated for 30 min at room temperature. Afterwards, the sepharose beads were pelleted again and 50 µl of the supernatant were mixed

with 50 µl medium (1:50 final dilution). Then, the neutralization assay was carried out as described earlier.

#### *Detection of SV40 DNA in serum*

Frozen serum samples were shipped on dry ice to the St. Olavs Hospital, Norway, and analyses were conducted at the Department of Pathology and Medical Genetics. Proteinase K (QIAGEN, Hilden, Germany) was used for protein digestion and DNA-extraction by Gene Vision BioRobot M48 (QIAGEN), using the standard protocol of MagAttract<sup>®</sup> DNA M48 kit (QIAGEN, West Sussex, UK). Fifty microliter DNA eluate was obtained from each sample. Primers were designed to amplify 239 bp of the SV40 enhancer region containing the double 72 bp repeat (SV40en1F: 5'-aac tgg ggc gag tta ggg, SV40en2R: 5'-tac ctt ctg agg cgg aaa ga) and 257 bp of the C-terminus of the large T-antigen (SV40Tcend2F: 5'-ggg agg tgt ggg agg ttt t, SV40Tcend2R: 5'-atg tgg cta tgg gaa ttg ga).

PCR reactions were performed in 25 µl reaction volume with 5 µl DNA eluate containing 50–100 ng total DNA and Gold Buffer (Applied Biosystems, Foster City, CA), dNTP, primer, AmpliTaqGold<sup>®</sup> DNA Polymerase (Applied Biosystems) and MgCl<sub>2</sub> following the standard protocol. After denaturation (95°C, 7.5 min) DNA was amplified in 40 cycles (95°C, 30 sec; 56°C, 30 sec; 72°C, 30 sec) followed by final elongation (72°C, 7 min). The size of PCR-products was determined by electrophoresis in 4% agarose minigels (E-gel, Invitrogen, Carlsbad, CA.). Twenty base pair DNA ladder (Sigma-Aldrich, St. Louis, MO) was used as size marker. Positive control DNA from a human osteoblast cell line transfected with SV40 large T-antigen (hFOB 1.19, ATCC number CRL-11372) and water as negative control were included in all PCR assays. Analytical sensitivity with hFOB 1.19 DNA was 5 pg for the SV40 enhancer and 0.5 pg for the SV40 Tag C-terminus, corresponding to DNA of 1 and 0.1 cell, respectively, necessary for SV40 sequence detection. PCR-products were cut out of the gel and extracted according to the MinElute Gel Extraction Kit Protocol (QIAGEN, Hilden, Germany). DNA sequencing was performed with the CEQ 8,000 sequencing system (Beckmann Coulter, Fullerton, CA).

#### *Statistical methods*

Antibody levels were measured on a continuous scale and used as dichotomous variables in the statistical analyses. Box-plots were produced to visualize the distribution of antibody levels for cases and controls separately, and *t*-tests were run for formal comparison of the distributions. To evaluate cross-reactivity between the virus markers, Spearman's correlation coefficients between the 6 antibodies to the 3 polyomaviruses included were calculated. To estimate the risk of mesothelioma conditional logistic regression was used to calculate the odds ratios (ORs) for being a case given a positive response to one of the selected viral antibodies. Ninety-five percent confidence intervals (95% CIs) were computed. Adjustment for age, gender, time of blood sampling and county was included in the matched design of the study. All ORs were additionally adjusted for asbestos exposure at 4 levels. The software program Stata<sup>45</sup> was used in all statistical analyses.

Approval from the ethical committee for medical research in Norway and permission from the Norwegian Data Inspectorate to perform the study was obtained.

## **Results**

Of the 49 mesothelioma cases included, 41 men and 8 women, 38 were pleural, 1 mediastinal, 1 pericardial, 7 peritoneal, 1 both pleural and peritoneal and 1 was located in the scrotum. Forty-one were epithelial, 1 sarcomatous and 7 mixed type. Year of birth ranged from 1923 to 1951 (mean 1938, interquartile range 1929–1947), blood samples were drawn between 1972 and 1991 (mean 1981) and cases were diagnosed between 1980 and 2002 (mean 1996, interquartile range 1992–1999). Age at diagnosis ranged from 40 to 79 (mean 57, interquartile range 51–67). The time span

between serum sampling and cancer diagnosis ranged from 0.4–30 years, with a mean duration of 15 years (interquartile range 8–22 years). Only one case had less than 1 year between blood sampling and diagnosis.

Six (12.2%) of the cases and 17 (11.6%) of the controls were born 1949–1951 when vaccination with possibly contaminated vaccine was considered almost certain. Additionally, 20 (40.8%) cases and 61 (41.5%) controls were considered probably exposed (born 1938–1948). Among cases and controls, 8.2% and 2.0%, respectively, reported jobs entailing high or moderate exposure to asbestos at all 3 censuses. The prevalence of exposure to asbestos at any census was 26.5% among cases and 21.1% among controls. Mesothelioma risk for asbestos exposure was increased with an OR of 4.5 (95% CI 1.0–20.9) for those exposed at all 3 censuses compared to those never exposed.

Prevalence and level of VP1 antibody reactivity in the 196 sera analyzed was very high for BKV (94%, median reactivity of the positive sera 3,059 MFI) and JCV (77%, 4,029 MFI) (Figs. 1a and 1b). The majority (58%) of the sera also reacted with SV40 VP1, but levels of antibody reactivity were much lower (median reactivity of the positive sera 895 MFI).

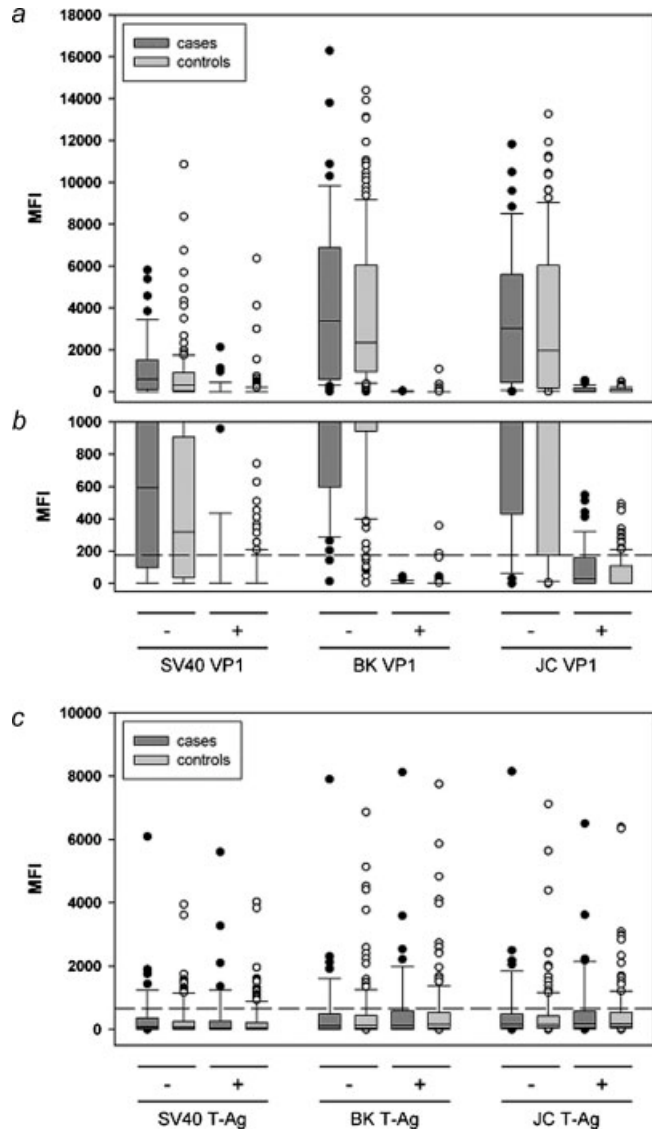
Blocking of BKV and JCV VP1 antibodies by serum preincubation with soluble MBP-VP1 fusion proteins of these 2 viruses drastically reduced BKV and JCV VP1 antibody levels (Figs. 1a and 1b). In the 196 sera analyzed the BKV/JCV VP1 block efficiently suppressed BKV VP1 antibodies, 99% of the 185 positive reactions were blocked by at least 95%, and 98% of the positive reactions were blocked below cut-off. For JCV VP1 antibodies the block was slightly less efficient, 90% of the 150 positive reactions were blocked by at least 95%, and 85% of the positive reactions were blocked below cut-off. The BKV/JCV VP1 block also drastically reduced antibody reactions with SV40 VP1, but to a lesser extent than with BKV and JCV VP1 (Figs. 1a and 1b). Of the 114 initially SV40 VP1-reactive sera only 81% could be blocked by 95%, 23 of the sera (20% of initially positive sera, 12% of all sera) remained above cut-off. Some of the remaining SV40 VP1 reactions were still rather strong: 10 out of 23 (43%) were above 3× cut-off, *versus* only 1 out of 3 (33%) and 1 out of 30 (3%) for BKV and JCV, respectively. These results indicate that the majority but not all of the SV40 VP1 antibody reactions in these human sera are due to BKV and JCV antibodies cross-reacting with SV40 VP1.

In the absence of the BKV/JCV VP1 block the SV40 VP1 antibody reactivities were slightly correlated with BKV VP1 and JCV VP1 antibodies, the Spearman's correlation coefficient  $\rho$  being 0.29 and 0.16, respectively (Table I). The SV40 VP1 antibody reactivity remaining after the BKV/JCV VP1 block was unrelated to BKV and JCV VP1 antibodies as shown by very low  $\rho$  values of  $-0.06$  and  $-0.03$  between the blocked SV40 VP1 antibodies and unblocked BKV and JCV VP1 antibodies, respectively, and was also unrelated to the blocked BKV and JCV VP1 antibodies ( $\rho$  0.08 and  $-0.05$ , respectively, not shown). The correlation between the blocked and the unblocked SV40 VP1 antibodies remained with a  $\rho$  value of 0.46 (not shown).

Addition of soluble SV40 VP1-MBP fusion protein to the BKV/JCV VP1 block solution abolished the remaining SV40 VP1 antibody reactivities for all sera except one, whereas addition of VP1-MBP fusion protein of the B-LPV, another primate polyomavirus distantly related to SV40, BKV and JCV, did not.

All 23 “nonblockable” sera that still reacted above cut-off with SV40 VP1 when preincubated with BKV/JCV VP1 were analyzed

for SV40 neutralizing activity, together with 8 “blockable” sera that reacted with SV40 VP1 when preincubated with HPV16 L1 (median 1,450 MFI, range 1,109–4,939 MFI), but not anymore (0 MFI) when preincubated with BKV/JCV VP1, and 8 “non-reactive” sera that did not react with SV40 VP1 (0 MFI) when



**FIGURE 1** – Distributions of antibody reactivities against SV40, BKV and JCV VP1 (a), bottom enlarged in (b) and Tag in mesothelioma cases ( $n = 49$ ) and controls ( $n = 147$ ) with (+) and without (–) preabsorption of antibodies to BKV and JCV VP1 (c). Boxes encompass 25 and 75 percentiles with medians indicated by horizontal bars, whiskers indicate 10 and 90 percentiles. Filled (cases) and empty (controls) circles indicate individual outliers, cut-offs used to distinguish antibody-positive and -negative values are shown as dashed horizontal lines (175 and 650 MFI for VP1 and Tag, respectively).

**TABLE I** – SPEARMAN'S CORRELATION COEFFICIENTS FOR THE ESTIMATES OF ANTIBODIES TO THE POLYOMAVIRUSES ( $p$ -VALUES)

	SV40 VP1 unblocked	SV40 VP1 blocked	BKV VP1 unblocked	JCV VP1 unblocked	SV40 Tag unblocked	BKV Tag unblocked
BKV VP1 unblocked	0.29 (0.00)	$-0.06$ (0.41)				
JCV VP1 unblocked	0.16 (0.00)	$-0.03$ (0.71)	0.02 (0.77)			
SV40 Tag unblocked	0.38 (0.00)	0.07 (0.31)		0.08 (0.29)		
BKV Tag unblocked	0.15 (0.00)	$-0.4$ (0.61)	0.27 (0.00)	0.09 (0.22)	0.67 (0.00)	
JCV Tag unblocked	0.23 (0.00)	$-0.00$ (0.95)	0.32 (0.00)	0.11 (0.11)	0.68 (0.00)	0.78 (0.00)

preincubated with HPV16 L1. Five neutralizing sera were identified, all of which belonged to the group of the "nonblockable" sera. Four of these were positive in 2 out of 2 assays, 1 serum (no. 143) was tested only once (1/1). The 5 neutralizing sera were preabsorbed with HPV16 L1, BKV/JCV VP1 or SV40 VP1, and then analyzed again for SV40 neutralization. Four of the 5 sera showed neutralizing activity also when preincubated with HPV16 L1, 1 serum (no. 143) failed to reproduce the neutralization activity observed in the screening assay. All 5 sera did not exhibit neutralizing activity anymore when preabsorbed with either BKV/JCV VP1 or SV40 VP1, respectively.

Antibody reactivities to Tag of the 3 polyomaviruses were much less prevalent (15% for SV40, 19% for BKV and 19% for JCV) than to the VP1 proteins (Fig. 1c). The BKV/JCV VP1 block had no significant effect on Tag antibodies. SV40 Tag antibodies were correlated with those against BKV and JCV Tag ( $\rho$  0.67 and 0.68, Table I) indicating a substantial cross-reactivity.

The distributions of antibody responses in cases and controls are shown as box-plots in Figure 1 and results of the multivariate analyses of mesothelioma risk for the presence of positive antigen response in Table II. Only results adjusted for asbestos exposure are given, although this adjustment did not change the overall results essentially. No significant associations with any viral antibody response were seen. All but one of the effect estimates were above unity, the highest OR being associated with high ( $> 3^*$  cut-off) level of SV40 antibody response in the presence of BKV/JCV VP1 block. When the subgroup of cases and controls probably or certainly vaccinated with contaminated vaccine was analyzed, there was also no significant association with antibodies against the SV40 antigens although the OR associated with high titers against SV40 VP1 was 3.2 (95% CI 0.8–13.0).

Most of the serum samples gave no SV40 PCR products; only few resulted in a PCR product (25 of the enhancer and 7 of the C-terminus) detectable by electrophoresis and ethidium-bromide staining. DNA sequencing of these products showed no SV40 or SV40-related sequences, therefore the products were classified as unspecific.

## Discussion

This is the first study to investigate the association of prediagnostic SV40 markers to subsequent risk of mesothelioma, and to apply a panel of serological assays on the same serum samples. The cases and controls were selected from a well-defined study base, the Janus Serum Bank. The ascertainment of cancer diagnoses was based on the Cancer Registry of Norway, considered to be virtually complete on solid tumors. All cases were histologically verified at the time of diagnosis, and an additional extensive immunohistochemical investigation was performed in conjunction with the present analysis. Selection of controls from the same study base that have yielded the cases ensured an unbiased com-

parison group. A major limitation of the study is the small sample size. However, the number of cases is the total of verified mesothelioma cases with available prediagnostic serum that have arisen in Norway since 1972. Additional analyses were also done including the 20 cases lacking other verification than initial histology at the time of diagnosis, and their corresponding controls. These analyses did not give any materially different results.

Only 26% of cases were classified as asbestos exposed, which is much lower than what would be expected in light of the attributable fraction of 84% estimated previously.<sup>38</sup> This may be explained by the relatively crude asbestos exposure estimate based on census information from 1 to 3 points in time. However, the risk estimate of 4.5, associated with the highest exposure level, was approximately as expected and comparable to what has been seen in other studies.

One major question in SV40 epidemiology is the validity of the antibody measurements. Recently published studies have shown that SV40 reactivity in human sera may be almost entirely a result of cross-reactivity with BKV and JCV antibodies.<sup>24,25,46</sup> Also in the present study, when SV40 VP1 antibodies were measured in the antibody capture assay without precautions to suppress cross-reactive BKV and JCV VP1 antibodies they were correlated with BKV and/or JCV VP1 responses.

To eliminate BKV and JCV VP1 antibodies that might cross-react with SV40 we developed a potent blocking assay based on soluble BKV and JCV VP1 proteins over-expressed in bacteria in fusion with MBP. The efficiency of the combined BKV/JCV VP1 block was demonstrated here by the strong, almost complete inhibition of all BKV VP1 responses and most of the JCV VP1 responses. As expected for cross-reactive antibodies also SV40 VP1 reactivities were strongly suppressed by the BKV/JCV VP1 block, however the SV40 reactivity of a substantial fraction of sera (12% of all sera and 20% of initially positive sera) could not be completely inhibited. Also, 10 (43%) out of the 23 SV40 VP1 reactivities remaining in the presence of the block were strong ( $> 3^*$  cut-off) *versus* only 1 out of 3 (33%) and 1 out of 30 (3%) for BKV and JCV, respectively. These remaining SV40 VP1 antibody reactions could represent true SV40 infections in humans or could still be cross-reactive antibodies resulting from infection with another, yet unidentified human polyomavirus. During development of the BKV/JCV VP1 block we observed that use of VP1 of only 1 of the human polyomaviruses in the blocking buffer was rather inefficient in blocking SV40 VP1 reactivities (Waterboer T, Rizk R, Pawlita M, unpublished observation) indicating that different human polyomaviruses can induce cross-reactive antibodies to different epitopes on SV40 VP1.

The prevalence of SV40 VP1 antibody reactivities in the antibody capture assay under BKV/JCV VP1 block conditions was 10.9% among controls, but was reduced to 4.1% when the higher cut-off point was chosen. These prevalences are higher than would be expected compared to previous studies, which however used

TABLE II – PROPORTION OF CASES AND CONTROLS WITH POSITIVE ANTIBODY RESPONSE AND ODDS RATIOS (OR) OF MESOTHELIOMA FOR THE PRESENCE OF ANTIBODIES TO SELECTED VIRAL PROTEINS WITH CORRESPONDING 95% CONFIDENCE INTERVALS (95% CI)

Virus marker	Positive antibody response (n)	Positive antibody response (%)		Whole sample (49 cases/147 controls)		Born 1938–195126 (cases/78 controls) <sup>1</sup>	
		Cases	Controls	OR	95% CI	OR	95% CI
SV40 VP1 unblocked	114	65.3	55.8	1.5	0.8–2.9	1.0	0.4–2.2
SV40 VP1 blocked	23	14.3	10.9	1.5	0.6–3.7	1.4	0.4–4.7
SV40 VP1 blocked and high titer <sup>2</sup>	10	8.2	4.1	2.0	0.6–7.0	3.2	0.8–13.0
SV40 VP1 unblocked and neutralized <sup>3</sup>	5	2.0	2.7	0.8	0.1–6.7	1.0	0.1–9.6
SV40 VP1 blocked and neutralized <sup>3</sup>	0	0	0	–	–	–	–
BKV VP1		95.9	93.9	1.7	0.3–8.4	1.7	0.2–14.5
JCV VP1		81.6	74.8	1.5	0.6–3.7	2.1	0.6–7.4
SV40 Tag		18.4	14.3	1.4	0.6–3.2	0.7	0.2–2.5
BKV Tag		22.5	18.4	1.4	0.6–3.0	0.9	0.3–2.6
JCV Tag		18.4	19.1	1.0	0.4–2.2	0.3	0.1–1.4

<sup>1</sup>Certainly or probably vaccinated, according to vaccination program. <sup>2</sup>MFI  $> 3^*$  cut-off. <sup>3</sup>All previously positive and a selection of negative samples retested, total sample included in statistical analysis.



different assays and different cut-off definitions. Knowles *et al.*, using a micro well SV40 neutralization assay without BKV or JCV block, described an SV40 seroprevalence in human sera of 3.2%.<sup>46</sup> Carter *et al.*, using a VLP-based ELISA found 6.6% SV40 seroprevalence, but none of the positive reactions remained after competition with BKV or JCV VLP.<sup>25</sup> In accordance with this, in the present study only 5 positive samples remained when all previously positive samples and a selection of negative samples were retested in a neutralization assay, and none of these could neutralize SV40 when BKV/JCV was blocked.

Even if the SV40 VP1 antibody response measured while blocking the activity of BKV and JCV VP1 antibodies were taken to represent true SV40 infection, no large differences between cases and controls are seen in the overall analyses, indicating no association between SV40 infection and subsequent mesothelioma risk. The results from the additional neutralization assays strongly support this conclusion. This may imply that the frequent finding of SV40 DNA fragments in mesothelioma tumors either is caused by enhanced viral replication favored by the disease process,<sup>47</sup> or that it simply stems from contamination.<sup>10,11</sup>

The study was designed on the assumption that SV40 in humans induces persistent infections with a continuous and relatively high production of antibodies as it does in monkeys<sup>24</sup> and similar to the serum responses seen with BKV and JCV infections in humans. Antibodies to VP1 are then expected to be a marker of acute or persistent infection. However, this may not be the case for SV40. Relatively little is known about SV40 infections in human populations. Early serological studies have indicated that individuals who received SV40-contaminated vaccines may develop moderate to high titers of SV40 neutralizing antibodies.<sup>19,48</sup> But these titres may not persist over decades. In the present study all serum samples were collected at least 10–15 years after potential exposure. Several studies of recently collected sera from age groups probably vaccinated with SV40-contaminated preparations also have not found high-titre SV40.<sup>21,25,46,49–51</sup> It should be noted, however, that all comparisons with previous serological results might be dubious because of the difficulties encountered with cross-reactivity of antibodies.

It is also possible that antibodies to Tag are produced only during the acute infection and then later as the carcinogenic process is active. Thus, if there were an association with cancer risk, Tag may not be expressed in sufficient amounts for the detection of a measurable antibody response before a tumor mass of a reasonable size had developed. This means that the absence of an association of SV40 Tag antibodies as seen here in prediagnostic sera of mesothelioma patients cannot rule out a potential association of SV40 with mesothelioma. The SV40 specificity of the Tag antibody

reactivities presented here is limited since these antibodies are highly correlated with BKV and JCV Tag antibodies and in contrast to the VP1 antibody analyses so far no blocking system has been developed to suppress the presumed SV40 Tag cross-reaction of BKV and JCV Tag antibodies.

Even if no true SV40 antibody response was seen, it may be of interest to note that the ORs for all serological markers, *i.e.* also BKV and JCV tend to be elevated above unity, even if nonsignificantly so. In most studies, only single antigens are being studied. Here, a series of related viral proteins have been investigated. It would be of interest to test larger mesothelioma samples and also other cancer types, to investigate whether this higher seroreactivity is a consistent feature of mesothelioma carcinogenesis or even a more general phenomenon related to cancer development.

The analyses of SV40 DNA sequences in serum yielded no positive results. Positive and negative controls were always included and yielded the expected positive and negative PCR results. Any presence of viral fragments many years after an infection in cases and not in controls would have strongly supported the hypothesis of a role of SV40 in carcinogenesis. The absence of these markers, though, cannot be taken as strong evidence for the opposite, since viral fragments tend to be cleared from the circulation after the acute infection. In a recent study on BKV and neuroblastoma no BKV-DNA was detected in serum in spite of a seroprevalence of 83% in cases and 80% in controls.<sup>52</sup>

## Conclusion

In the present study, a panel of serological assays was applied on preclinical serum from mesothelioma cases and controls to identify a possible association with SV40 infection. With increasing specificity of the assays used, the prevalence of SV40 positivity decreased, so that no truly SV40-antibody positive samples were identified among cases and controls. No significant association between SV40 antibody response in prediagnostic sera and risk of mesothelioma was seen. Nonsignificant, higher reactivities were observed among the cases for all 3 polyomaviruses, indicating a possible prediagnostic difference between cases and controls that may warrant further investigation.

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## CHAPTER FOUR

### **3rd Contribution**

**Humoral immune response in women with high grade cervical  
intraepithelial neoplasia (CIN II/III) after vaccination with HPV16  
L1/E7 chimeric-virus-like particles (cVLP)**

Rizk *et al.*, submitted to a scientific Journal

**Humoral immune response in women with high grade cervical intraepithelial neoplasia (CIN II/III) after vaccination with Human papillomavirus type 16 L1/E7 chimeric-virus-like particles (cVLP)**

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## Abstract

High-risk human papillomavirus types are the main etiologic agent of cervical cancer and its precursor lesions. Many clinical and preclinical prophylactic vaccine trials were conducted using virus-like particles (VLP) which resemble the native virions physically and immunologically. Chimeric VLP (cVLP) consisting of C-terminally truncated L1 protein fused to sequences of the HPV16 E7 oncoprotein have been recently used in a combined protective and therapeutic vaccine trial (*Kaufmann et al 2007*). CVLP vaccine showed a good safety profile and could induced HPV 16 L1 as well as E7 antibodies. In this study, we aimed to analyze this humoral immune response in detail.

The vaccination target group was women diagnosed with cervical intraepithelial neoplasia (CIN) grade II or III. The vaccine was given either in a low- (75 µg) or a high-dose at weeks 0, 2, 6, and 12. Blood samples for serology were collected at weeks 0, 2, 4, 8, 14, and 24. and assayed for induced antibodies using multiplex serology and pseudovirion-based neutralization assay.

Of the study participants, 58% and 17% had naturally induced HPV16 L1 and E7 antibodies at study entry, respectively. 44% had neutralizing antibodies. None of the placebo recipients showed any changes in the immune response over time. Upon vaccination, HPV 16 L1 antibodies were induced in all patients, with titers up to 200,000 in both vaccine groups. L1 neutralization titers ranged from 60 to 5400 in the low-dose and up to 48,600 in the high-dose group. Response to E7 was generally weaker. Cross-reactivity to L1 as well as to E7 was very frequent with the closely related HPV 31, 35 and 33 types, however, less frequent cross-reactivity to L1 of alpha cutaneous and skin types was observed. No cross-neutralization to HPV18 or 11 was induced. Antibodies against 16E6, E4, E2 and E1 were also induced.

In summary, HPV16L1/E7 cVLP vaccine is highly immunogenic and might be protective also against very closely related HPV types. However, the strength of the developed response correlates with both the given dose- and the immune status before vaccination.

**Keywords:** Cervical cancer; HPV; cVLP; therapeutic vaccination; multiplex serology; antibody; neutralization.

## 1. Introduction

High-risk human papillomavirus (HPV) types are considered the major etiologic factor of invasive cervical cancer and its precursor lesions in women (Fehrman & Laimins, 2003; zur Hausen, 2002). HPV type 16 accounts for 50-60% of the cervical cancers and high-grade cervical intraepithelial neoplasias (CIN II and III) and 35% of all low-grade lesions (CIN I) in most countries (ALTS, 2000; Bosch *et al.*, 1995; Kulasingam *et al.*, 2002; Moscicki *et al.*, 2001), followed by HPV types 18 (10-20%) and 31 and 45 (4-5% each) in cervical cancers (Bosch & de Sanjose, 2003; Walboomers *et al.*, 1999). Ten further, less prevalent types are also classified as HR-HPV (Bosch & de Sanjose, 2003; Clifford *et al.*, 2003a; Clifford *et al.*, 2003b).

Every year, around 500,000 women develop invasive cervical cancer and approximately 275,000 deaths per year are recorded (Parkin *et al.*, 2005).

In comparison to most viral infections, the natural immune response to HPV after infection is weak and slow (Frazer, 1996). More than 80% of HPV infections are spontaneously cleared (Ho *et al.*, 1998), possibly due to cellular immune response (Frazer, 2004). However, inadequate immunologic control of the infection and different viral evasion mechanisms (O'Brien & Saveria Campo, 2002) may contribute to virus persistence and the development and progression of premalignant cervical lesions (Frazer, 1996; Tindle, 1996).

The most readily detectable HPV-specific immune response is antibody formation against the major capsid protein L1. These antibodies appear between four months to five years after the first infection. However, 50% of HPV16-infected women do not develop any measurable capsid-specific antibody response (Carter *et al.*, 2000).

L1-specific antibodies are considered markers for current or previous infection (Carter *et al.*, 1996; Carter & Galloway, 1997; Kirnbauer, 1996) as they occur more frequently in patients with persistent infections or CIN III lesions than in cervical cancer patients (Nonnenmacher *et al.*, 1995) and they are highly-type specific. On the other hand, antibodies to the early, non-structural proteins E6 and E7 that are consistently overexpressed in HPV-transformed cells, have been found to be strongly associated with cervical carcinoma and are therefore markers of malignancy (Lehtinen *et al.*, 2003; Meschede *et al.*, 1998; Silins *et al.*, 2002; Zumbach *et al.*, 2000). The cellular immune response to the E6 and E7 oncoproteins seems to be more important for clearance of the infection than the humoral immune response.

The development of serological assays to analyze the immune response after infection with HPV faces some limitations including the large number of HPV types associated with different diseases and difficulties in isolating or producing sufficient quantities of infectious virions. HPV L1 protein has the intrinsic capacity to self-assemble into virus-like particles (VLP) (Hagensee *et al.*, 1994; Kirnbauer *et al.*, 1993; Zhou *et al.*, 1991) which mimic the natural virions morphologically, display immunodominant conformational epitopes (Christensen *et al.*, 1996a; Christensen *et al.*, 1996b; Christensen *et al.*, 2001) and are able to generate high titers of type-specific neutralizing antibodies (Schiller 1996). HPV16 VLP-based serological assay was first established in 1994 (Kirnbauer *et al.*, 1994) and then applied to other HPV types.

Improvements in serological techniques include capture assays using bacterially expressed recombinant viral proteins (Sehr *et al.*, 2001; Sehr *et al.*, 2002) and the introduction of fluorescent bead-based technologies (Luminex) (Waterboer *et al.*, 2005; Waterboer *et al.*, 2006). Glutathione-S transferase (GST) L1 fusion proteins were found to maintain the immunogenic properties of the virus particle and VLP, including presentation of neutralizing, linear and conformational epitopes (Yuan *et al.*, 2001). Many systems were also established to develop an applicable HPV neutralization assay using athymic mouse xenografts (Kreider *et al.*, 1987), raft culture systems (Meyers & Laimins, 1994) or producing infectious HPV pseudovirions *in vitro* (Roden *et al.*, 1996; Touze & Coursaget, 1998; Unckell *et al.*, 1997). The most recent *in vitro* neutralization assay is based on pseudovirions carrying a secreted alkaline phosphatase (SEAP) reporter gene (Buck *et al.*, 2005; Pastrana *et al.*, 2004).

Recently, many trials with highly effective protective vaccines have been conducted to prevent premalignant and malignant diseases related to infection with high-risk HPV types.

Early preclinical trials using animal models showed that the VLP used were protective both against cutaneous warts as those induced by the cottontail rabbit papillomavirus (CRPV) (Breitburd *et al.*, 1995; Jansen *et al.*, 1995) and also against mucosal warts induced by the canine oral papilloma virus (COPV) (Suzich *et al.*, 1995) and the oral bovine papilloma virus 4 (BPV 4) (Kirnbauer *et al.*, 1996). In these models, the vaccines used induced high-titers of serum neutralizing antibodies and protected against experimental challenge with the infectious virus.

In humans, the first HPV16 VLP (Harro *et al.*, 2001) and HPV 11 VLP (Evans *et al.*, 2001) phase I vaccine trials showed that the vaccines were well tolerated and highly immunogenic even when administered without adjuvant and that the antibody titer in the immunized subjects was at least 40-folds higher than in natural infections. Recently, several clinical

phase II and III randomized placebo-controlled trials have been conducted. The vaccines used were either monovalent HPV 16 (Koutsky *et al.*, 2002; Mao *et al.*, 2006; Poland *et al.*, 2005), bivalent HPV 16/18 (Harper *et al.*, 2004; Harper *et al.*, 2006), or quadrivalent HPV 16/18/6/11 (Villa *et al.*, 2005; Villa *et al.*, 2006a). The vaccination results showed that these vaccines were well tolerated, highly immunogenic as they induced type-specific neutralizing antibodies, provided complete protection against persistent type-specific infections and HPV associated diseases and induced long-term immunity for up to 4.5 years (Harper *et al.*, 2006; Villa *et al.*, 2006b).

Two vaccines are now commercially available, Glaxo Smith Kline's (GSK) bivalent vaccine named Cervarix<sup>TM</sup> and the Merck quadrivalent vaccine Gardasil<sup>TM</sup>. Phase III trials of these two vaccines revealed that they were highly efficacious in preventing HPV 16/18 related CIN II/III and adenocarcinoma in situ (ASI) (FUTUREII, 2007; Garland *et al.*, 2007; Paavonen *et al.*, 2007) and high grade vulval and vaginal lesions associated with HPV 16 or HPV 18 infection (Joura *et al.*, 2007).

In contrast to prophylactic vaccines which aim predominantly at inducing the humoral immune response, therapeutic vaccines aim at stimulating the cell-mediated immune response. Since VLP were unable to cause regression of established lesions, they are not suitable for therapeutic applications. Therapeutic vaccines focus mainly on HPV oncoproteins E6 and E7, as their continuous expression in infected cells is essential for the development and maintenance of precancerous lesions and their progression into invasive cancer (zur Hausen, 2002). HPV E7 protein is considered the better candidate for a therapeutic vaccine than E6 as it is more strongly conserved (Zehbe *et al.*, 1998) and well immunologically characterized. Chimeric VLP (cVLP) are generated by the fusion of E7 to L1 (Muller *et al.*, 1997) and when used for immunization, they were able to induce L1-specific and neutralizing antibodies, to elicit an E7-specific cellular immune response and to prevent the growth of E7-positive cells in mice (Greenstone *et al.*, 1998; Nieland *et al.*, 1999; Schafer *et al.*, 1999) and after *in vitro* vaccination of human cells (Kaufmann *et al.*, 2001).

Recently, a randomized, double-blind, placebo-controlled phase I clinical vaccination trial with HPV16 L1/E7 cVLP has been conducted (CT 1006; sponsored by MediGene, Martinsried, Germany). The target group consisted of patients with HPV 16 positive CIN II/III. The first results demonstrated evidence for the safety of the vaccine and a non significant-trend for clinical efficacy. The trial showed that the vaccine is able to induce L1 and E7 specific antibodies and also a cellular immune response (Kaufmann *et al.*, 2007).

The aim of the current study is to analyze the details of the humoral immune response induced after vaccination with HPV16 L1/E7 cVLP. We analyzed the induction of HPV16 L1-specific as well as E7-specific antibodies. Moreover, we investigated the induction of L1 or E7 cross-reactive antibodies to other HPV types, induction of antibodies to other HPV 16 early proteins (E6, E4, E2 and E1) and finally whether the particles are able to induce protection by neutralizing antibodies.

## **2. Methodology**

### **2.1 Study design and vaccine**

The present 24 week, multi-center, double-blind, placebo-controlled phase I immunogenicity trial was sponsored by MediGene (Martinsried, Germany). Serological analyses presented here were conducted at the German Cancer Research Center in Heidelberg, Germany.

Thirty-six women histologically diagnosed as CIN II or III patients were recruited for this study. They were all HPV 16 DNA positive and negative to any other high-risk type. Details about the selection criteria and disease profiles of these subjects were previously published (*Kaufmann 2007*). The same report describes in details the properties of the administered vaccine. Briefly, it consists of highly purified HPV 16 L1/E7 cVLP (*Mueller 1997*) given intramuscularly without adjuvant. The enrolled patients were divided randomly into three groups of each 12 patients. The first group received placebo, the second group received 75 µg (low-dose) and the third group received 250 µg (high-dose) of the vaccine. Four vaccine injections of the same dose were given at weeks 0, 2, 6, and 12. Blood samples for serology were collected at baseline and at weeks 2, 4, 8, 14, and 24.

### **2.2 Multiplex serology**

Specific antibody responses to HPV 16 L1 and E7 were analyzed. Additionally, L1-reactive antibodies to 27 other HPV types belonging to 17 different species were analyzed (HPV 31, 33, 35, 52, and 58 ( $\alpha 9$ ); HPV 18 and 45 ( $\alpha 7$ ); HPV 6 and 11 ( $\alpha 10$ ); HPV 2 and 57 ( $\alpha 3$ ); HPV 3, 10, and 77 ( $\alpha 2$ ); HPV 32 ( $\alpha 1$ ); HPV 49 ( $\beta 3$ ); HPV 15 and 38 ( $\beta 2$ ); HPV 92 ( $\beta 4$ ); HPV 5 ( $\beta 1$ ); HPV 4 ( $\gamma 1$ ); HPV 50 ( $\gamma 2$ ); HPV 48 ( $\gamma 3$ ); HPV 60 ( $\gamma 4$ ); HPV 41 (*nu*); HPV 1 (*mu1*) and finally HPV 63 (*mu2*)), as well as E7-reactive antibodies to 8 other types of 3 different species (HPV 31, 33, 35, 52, and 58 ( $\alpha 9$ ); HPV 18 and 45 ( $\alpha 7$ ) and HPV 6 and 11 ( $\alpha 10$ )). The detection assay used is based on expression of all L1 and E7 full-length proteins (with HPV



6b, 16, and 18 L1 lacking 10 amino acids at the N-terminus) as double fusion proteins with N-terminal Glutathione *S*-transferase (GST) and a C-terminal undecapeptide (tag) of the SV40 large T-antigen (*Sehr 2001, 2002*) in combination with multiplex bead-based technology (xMAP, Luminex Corp, Austin, Texas) (*Waterboer et al., 2005 and 2006*). Briefly, glutathione cross-linked to casein was cross-linked to different fluorescence-labeled polystyrene beads (SeroMap, Luminex, Austin, TX). Beads sets of different colors were individually loaded with different GST-tag fusion proteins. The beadmix was then incubated with serum diluted 1:100 for E7 assay and with three-fold dilutions starting at 1:100 for L1 antigens. Antibodies bound to the beads were stained by biotinylated anti-human-Ig and streptavidin-R-phycoerythrin. Beads were analyzed in a Luminex 100 analyzer that identifies the bead color and quantifies the antibodies bound to the viral antigens as previously described (*Waterboer 2005*).

### **2.3 Neutralization assays**

*In vitro* HPV 16, 18, and 11 neutralization assays based on HPV pseudovirions carrying a secreted alkaline phosphatase (SEAP) reporter gene were carried out as previously described (*Buck et al 2005, Pastrana et al 2004*). Screening for neutralizing antibodies was performed by infecting 300,000 293TT cells/ml with the different HPV pseudovirions in the presence of sera diluted 1:20. Infections were performed 5-6 h after seeding of the cells. Untreated cells, cells treated with pseudovirions alone or cells treated with pseudovirions in the presence of a known type-specific neutralizing serum were used as controls. Five days after infection, the supernatants were assayed for presence of SEAP using a chemiluminescent SEAP Reporter Gene Assay (Roche Diagnostics, Mannheim, Germany). When neutralizing, the endpoint neutralization titers were determined by titrating the neutralizing sera against the neutralized pseudovirus type.

### **2.4 Statistical analyses**

In all multiplex serology experiments, antibody reactivity against GST-tag without intervening viral sequence was used to define the background. The net median fluorescence intensity (MFI) was obtained by subtracting the background reactivity from the reactivity against the viral fusion proteins. Sera were classified positive at a given dilution if the net MFI value was greater than or equal to 150 and 50 in L1 and E7 serology, respectively.

In neutralization assays, net relative light units (RLU) were calculated by subtracting RLU of untreated cells (background) from RLU of cells treated with the serum/pseudovirion mixture.

The endpoint neutralization titer was defined as the last dilution yielding  $\geq 70\%$  reduction in the SEAP activity in comparison to the reactivity of the pseudovirions added without serum. The multiplex serology and neutralization titers are given as the reciprocal of the highest dilution showing positive signals for each assay.

Specific response due to vaccination was defined as a two-fold or higher increase in the antibody reactivity or titer in any week after vaccination relative to the response at baseline (W0). For L1, cross-reactivity was counted when titer difference was equal to three-folds in two consecutive sera or  $> 3$ -fold in at least one serum. For E7 cross-reactivity, two-folds or more differences in MFI values were considered significant.

The Wilcoxon signed rank sum test was used to compare the distribution of antibody titers or MFI between different timepoints within and between specific vaccine groups. The same test was also used to evaluate the cross-reactivity within or between the different vaccine groups. *P*-values  $< 0.05$  were considered statistically significant. Multiplex serology and neutralization assays were directly compared using Kappa statistics. All analyses were performed with SAS Statistical Package version (9.1).

### 3. Results

#### 3.1 Vaccination with HPV16 L1/E7 cVLP induces HPV16L1 antibodies

At baseline (week 0, W0), HPV16 L1 antibodies were present in twenty-one (58%) of the study participants. None of the placebo recipients seroconverted or increased the reactivity towards L1. All vaccinees showed an increased HPV16 L1 antibody response already at W2, visible both as strongly increased MFI values at 1:100 serum dilution and end-point dilution titer, 134-fold for the high-dose and 45-fold for the low dose group. ( $p < 0.001$  for both). There was a linear relationship of end-point titer (titers 100 to 81000) and MFI values at 1:100 (MFI 155 to 9344) (Figure 1, Table 1).

Among high-dose recipients the geometric mean titer did not change until W14 while low-dose recipients continued to increase antibody titer at lower rate, reaching similar level as the high dose group at W14. At W24 the titer decreased 2.5- to 3-fold in both groups (Figure 1C). Vaccinees baseline seronegative, at W2 reached a GMT of 2.2 only while the seropositives starting from 827 reached 67 (30-fold difference). At W14 the GMT in baseline seronegatives increased to 19.9 thus nearly reaching the GMT of 56.6 in the baseline seropositives (3-fold difference).

#### 3.2 HPV16 L1 neutralizing antibodies are induced upon vaccination

At baseline 16 participants (44%) had neutralizing antibodies (Table 1). None of the placebo recipients developed additional neutralizing antibodies. While 8 of the vaccinees developed a very weak response (border-line titers) over time, 16 showed strong response. For the later group, the increase in the neutralization titer was observed already at W2, the low-dose group reaching (GMT 661) a 1.8-fold lower level than the high-dose group (GMT 1159). At W14, the titer doubled among the low-dose recipients and tripled in the high-dose group; but at W24 already decreased 5- and 3-fold, respectively, in both groups (Table 1).

Vaccinees baseline neutralization negative, at W2 reached a GMT of 283 only while the seropositives starting from 104 reached 487. The titers and the group difference did not change at W4 and W8, but with the increase at W14 the titer among baseline negatives became slightly higher than among the baseline positives, at W24 both fell to very similar low levels (182 and 187, respectively).

Neutralization titers correlated with the Luminex titers ( $\kappa = 0.618$ ; 95% CI 0.304-0.932), indicating that in vaccinees Luminex titers are valid proxies for neutralizing antibodies

(Figure 2B). For natural antibodies, this correlation could not be applied (unpublished observation).

At baseline, three and one patients had natural neutralizing antibodies to HPV 11 and HPV 18, respectively. However, none of the vaccine recipients developed cross-neutralizing antibodies to HPV 11 or HPV 18 pseudovirions.

Neutralizing antibodies are expected to correlate most closely with the vaccine's potential for protection.

### **3.3 Vaccination induces L1 cross-reactive antibodies**

Although the vaccine used in this study was composed of L1 and E7 proteins of HPV 16, it was able to induce cross-reactive antibodies frequently to mucosal alpha types, and rarely to cutaneous alpha, beta, gamma, mu and nu types.

Induced cross-reactivity was observed in sera baseline seronegative to any other HPV types than HPV 16 and seroconverting after vaccination or baseline seropositive and increasing reaction.

In the placebo group, none of the patients showed any cross-reactivity. The reactions with other HPV types than 16 remained stable over time. All vaccinees seroconverted to at least one of the other HPV types analyzed here irrespective of vaccine dose (Figure 3A).

The kinetics of cross-reactive antibody formation was slightly delayed, especially in low-dose in comparison to that of HPV 16 L1 antibodies. The median time point of cross-reactivity seroconversions in patients of the high-dose group was W2 versus W4 in the low-dose group. At this week, the response plateau in most of the patients was reached.

Cross-reactivity titers (median 100, range 100 to 8100 in the low-dose group; median 100, range 100 to 218,700 in the high-dose group) were lower than HPV 16 specific titers (median 8100; range 100 to 218,700 in low-dose group versus median 24,300; range 900 to 218,700 in high-dose group). These differences were statistically significant ( $p < 0.050$  for all types) (Figure 3B, C).

Cross-reactions to alpha mucosal types were the strongest and most frequent in both groups. From the high risk types, HPV 31 followed by HPV 35 and 33 were the most frequent types (100% of seroconverted vaccinees). The least frequently recognized HR-HPV were HPV 52 and 58 (83%). Despite belonging to a different and phylogenetically distant species, cross-reactive antibodies to HPV 18 and 45 were also very frequent (100%) in both vaccine groups. In the high-dose group, the low-risk types HPV 11 and HPV 6 were less frequent than the high-risk types. In the low-dose group, frequency of HPV 11 was higher.

Generally, seropositivity to skin HPV type was least frequent (62% and 26% in high-dose and low-dose groups, respectively).

Cross-reactivity was more frequent in the high-dose recipients than in the low-dose recipients; the mean of the number of cross-reactive types was 19 and 14 in the both groups, respectively.

In conclusion, vaccination with HPV16 L1/E7 cVLP induces intra- and inter-species cross-reactive antibodies.

### **3.4 Vaccination induces HPV16E7 specific antibodies**

At study entry, only six patients (17%) had natural anti-HPV16E7 specific antibodies (median 110 MFI; range 51 to 636). The W0 HPV 16E7 response in the placebo recipients did not change over time. All vaccinees except one of the low-dose group developed an immune response to HPV16E7 (Figure 4A).

The median time of seroconversion in both vaccine groups was W2. The increase in HPV16E7 response in both vaccine groups continued after W2 (Figure 4B).

At any time after W2, reactivity to HPV16 E7 was stronger in high-dose recipients (GM 592 MFI; range 290 to 1222) than in low-dose recipients (GM 119 MFI; range 56 to 253). The differences between the two groups were statistically significant ( $p=0.003$ ) (Figure 4B). On the other hand, at W2, the response to HPV 16 E7 was 20- and 75-folds weaker than that observed for HPV 16 L1 response in term of MFI values in high-dose and low-dose groups respectively.

The immune response in patients who had natural HPV16E7 antibodies at W0 was 3.4-fold higher than in patients with no detectable natural antibodies at baseline.

### **3.5 Vaccination induces HPV16E7 cross-reactive antibodies**

Among the placebo recipients, two showed single seropositivity (HPV 33 and 45) at W2 or W24 with  $\geq 2$  folds response. Among vaccinees, HPV E7 cross-reactive E7 antibodies to at least one other type than HPV 16 were induced in 13 (54%, 5 low-dose and 8 high-dose) of the 24 vaccinees (Figure 5). Time of seroconversions was later (after W4) than for HPV 16 E7 antibody response (W2).

The response to any of the cross-reactive types (MFI median 154, max 3018, min 50) was weaker than to the specific immune response to HPV16E7 (MFI median 291, max 8207, min 51). However, only for HPV 35, differences in cross-reactivity strength between the two vaccine groups were statistically significant ( $p<0.02$ ). Multiple positivity was more frequent

in high-dose group than the low-dose group. Cross-reactivity followed the phylogenetic classification as most frequent cross-reactions (19% of all) were detected with the closely related HPV types of the  $\alpha 9$  species. It was more frequent in the high-dose group than in the low-dose group (Figure 5A). In the high-dose group, HPV 31 was the most frequent cross-reactive type (50%), followed by HPV 35 (42%) and HPV 33 (33%). The least cross-reactivity frequency was observed with HPV58 (17%). No cross-reactions to any  $\alpha 7$  or  $\alpha 10$  types were recorded. In the low-dose group, HPV 31 was again the most frequent cross-reactive type (18%). Frequencies for HPV 35, 33, 58, 45, and 6 were low (9% each). No cross-reactive antibodies to HPV18E7, 11E7 or 52E7 were detected.

### **3.6 Vaccination induces an immune response to other early HPV16 proteins**

While none of the placebo or high-dose groups were positive at study entry to any HPV 16 early proteins other than E7, two patients of the low-dose group were positive to both E2 and E4. The response to HPV 16 other early proteins among placebo remained stable over time. Upon vaccination, seven and six patients of the high-dose and low-dose groups respectively developed antibodies to at least one other early protein; E6, E2, E4 or E1 (Figure 6).

Time of seroconversion (W8 or later) in the three study groups to any of these early proteins occurred later than time of seroconversion of HPV 16 E7 or L1. Antibodies to HPV16 E6 were most frequent, followed by E4 and E2 and E1.

There were no statistically significant differences between the responses induced in both vaccine groups at W2 or later ( $p>0.05$ ).

#### 4. Discussion:

HPV16 is the most common associated type with cervical cancer (Bosch *et al.*, 1995; Walboomers *et al.*, 1999) and the most commonly detected HPV type in most epidemiologic studies (Cates, 1999; Koutsky, 1997). Several clinical trials have been conducted to evaluate HPV prophylactic vaccines consist of virus-like particles (VLP). As alternative to VLP, chimeric VLP were generated mainly to develop a vaccine that combines prophylactic and therapeutic properties. They are consisting of a C-terminally truncated L1 protein fused to sequences of the HPV16 E7 oncoprotein (Jochmus *et al.*, 1999).

This study aimed to analyze in detail the immune response after vaccination with HPV16L1/E7 cVLP vaccine (Kaufmann *et al.*, 2007). We investigated the induction of HPV16 L1 specific antibodies and also cross-reactive antibodies to L1 of other HPV types (n=27) including mucosal and skin types of different species (alpha 1, 2, 3, 7, 9, 10, beta 1, 2, 3, 4, gamma 1, 2, 3, 4, Mu 1, 2, and Nu) using Luminex multiplex technology (Waterboer *et al.*, 2005; Waterboer *et al.*, 2006). We also analyzed the type-specific and cross-reactive immune response to HPV16E7 oncoprotein. Additionally we extended the study to detect any immune response to other HPV16 early proteins including E6, E1, E4 and E2. Neutralizing antibodies against HPV16, 18 and 11 were analyzed using the in vitro neutralization assays based on HPV pseudovirions carrying a secreted alkaline phosphatase (SEAP) reporter gene (Buck *et al.*, 2005; Pastrana *et al.*, 2004).

The importance of this study is due to the fact that the patients received the vaccines are CIN II or III patients; so that we have data of antibody response to natural HPV infection. This allowed us to comment on the antibody response following immunization relative to that seen after acquisition of an HPV infection. All responses after vaccination were only due to the vaccine and not to any new infection. This is because antibodies due to a new infection need a long time to be detected in the patient serum.

As previously described (Jochmus *et al.*, 1999), cVLP can function as an intrinsic adjuvant. This give the current vaccine the advantage of being administered without use of any adjuvant in contrast to many other vaccine trials done so far; thus the immune responses detected after vaccination is only due to the vaccine components.

In general, virus-like particles (VLP)-based vaccines are thought to be safe. These particles are morphologically and immunogenically similar with the natural HPV virions, but contain no DNA which makes them non-infectious.

Although all the patients enrolled in this study are infected with HPV16, but for some patients no HPV16 antibodies were detected. It might be that these women had lost the antibody responsiveness either due to the loss of L1 expression following integration of the viral DNA into the host cell genome, or because of changes in immune function resulting from lesions formation or therapy.

HPV16L1/E7 cVLP vaccine is highly immunogenic. All participants who received the vaccine developed high titers of HPV16 L1 specific antibodies (around 100 folds or higher than what observed in natural infection represented by W0) out to week 24. These results are in agreement with previous clinical trials based on VLP-vaccines (Fife *et al.*, 2004; Harper *et al.*, 2004; Koutsky *et al.*, 2002; Villa *et al.*, 2005; Villa *et al.*, 2006a).

So far, neutralization assays are often considered to be the “gold standard” in assessing the immunogenicity of a prophylactic vaccine, so that the clinical significance of any vaccine trial is related to its ability to induce neutralizing antibody response. The data presented here showed that all of the HPV16L1/E7 cVLP vaccine recipients induced neutralizing antibodies giving it the properties of a successful prophylactic vaccine.

Data showed that neutralizing antibodies can be detected when the patient has a strong MFI signals (>450-700) giving an indication that Luminex is more sensitive than neutralization assay. However, technically there were a good correlation between HPV 16L1 Luminex titers and neutralization titers at 1:100. Such a correlation gave us the opportunity to analyze the possibility of using the Luminex readout MFI values at 1:100 as a surrogate to define or calculate the antibody and /or neutralizing titer of any serum, for example in case of having a limited volume of the serum for such analyses. Moreover, this correlation held for individuals as well as for the two vaccine groups, implying again that the Luminex appears to represent an appropriate surrogate assay for the neutralization assay.

Although all the vaccine recipients developed specific HPV16 L1 antibodies by two weeks after the initial immunization, but two factors had impact on the strength of the immune response developed. First, the dose of the given vaccine correlates with the reported immune



response. When a high dose (250µg) of the vaccine was given, the antibody titers were very high; all the baseline seronegative patients became positive and reached saturation at week 2 post vaccination. In contrast the response was lower (2-4 folds) and slower when less vaccine dose (75µg) was given. In this group, the antibody saturation was reached later at W14. A similar correlation has been described previously (Fife *et al.*, 2004).

A second factor was the immune status of the patients before receiving the vaccine. It seems that in CIN II or III patients, preexisting of HPV 16L1 antibodies at baseline influences the immune response after vaccination. Other clinical vaccine studies (Harro *et al.*, 2001; Koutsky *et al.*, 2002) have reported a similar finding; increase in antibody titers in subjects who were seropositive before vaccination. This might be due to formation of memory immune response to L1 capsid protein which helps in responding quickly to a new infection (cVLP particles in the vaccine). This memory immune response has been discussed in details by Olsson and colleagues (Olsson S. E. *et al* 2007).

In comparison to the response detected by Luminex, only the vaccine dose had a significant influence on the neutralizing antibody titers as higher vaccine dose resulted on higher neutralizing titers (up to 3,4 fold differences).

A wide inter- and intra- cross-reactivity patterns were reported by Luminex as many of the vaccine recipients had L1 antibodies to at least 7 types other than HPV16. This was expected since GST-L1 fusion proteins which are used as Luminex antigens have been shown to exhibit cross-reactive and non-neutralizing linear epitopes as well as conformational epitopes (Rizk *et al* 2007). Cross-reactivity follows loosely the phylogenetic grouping. The most frequent cross-reactivity was with the closely related HPV types in species alpha 9, followed by alpha 7 and alpha 10. However, some patients also cross-reacted with non-related HPV types including the cutaneous types.

In contrast, no cross-neutralizing antibodies have been detected after vaccination; which is also in agreement with the fact that neutralizing antibodies are directed against L1 conformational epitopes that are mostly type-specific. Based on these findings, we think that neutralization assays could be more genotype-specific than luminex. For example, sera from patients with very high HPV16L1 antibody titers did not neutralize HPV18 or HPV11 pseudovirions, although the same sera were cross-reactive in Luminex with both HPV types.

Cross-reactivity when present correlates with the number of vaccine doses; the more the doses the patient receives the more cross-reactivity to be observed. Moreover, HPV 16 L1 specific titers were higher than the cross-reactive titers ( $\geq 100$  fold differences). This observation has

been previously described (Wideroff *et al.*, 1995). This might be because upon every vaccine dose administered, the cVLP are accumulated and possibly increase the chance of occurrence of cross-reactive epitopes that may be present on the surface of other HPV types.

So far, cVLP in mice were found to induce HPV16E7-specific antibodies (Muller *et al.*, 1997). In this clinical study, vaccination with cVLP induced HPV16E7 specific antibodies as well. The responses in comparison with HPV16L1 antibodies were lower. A previous clinical study used HPV16E7 protein-based vaccine showed also that HPV16E7 antibody titers were low (Hallez *et al* 2004). The reason for lower E7 antibody response could be that L1 in the particles is present in a higher proportion than E7 or it is more exposed to the immune system. E7 cross-reactivity followed exactly the phylogenetic grouping. All cross-reactions were within types of the species alpha 9. Only in one case, a cross-reaction with HPV6 (alpha 10) was observed, however the response was extremely weak and just above the cut-off used to differentiate between positivity and negativity. Not all patients exhibited cross-reactive L1 antibodies showed also cross-reactivity to E7. The different structural properties and size of both proteins may explain why cross-reactivity to L1 was more frequent than to E7. As for L1, cross-reactivity to E7 correlated with the number of vaccine doses given to the patients.

The peak of antibody response after administration of the cVLP vaccine was reached directly after the first vaccination dose (at W2) followed by stable response until W14 where a slight increase in the response is observed (for neutralizing antibodies the response at W14 was very strong forming a second clear immune response peak). Afterwards a decline in the antibody level was generally observed. Serum antibody responses in baseline seropositive patients increased faster and peaked at higher levels than the responses in baseline seronegative patients. This response pattern is highly suggestive of an anamnestic response to the HPV16 antigens (L1 and E7). Typically, the immune responses decrease with time after antigen stimulation because absence of the antigen leads to removal of the stimulus for further antibody production. Moreover, plasma cells have a limited lifespan which means that production of antibodies would only due to presence of memory B cells (Olsson *et al* 2007). Unfortunately, we were unable to analyze the persistent of the induced antibodies after 24 weeks due to the short study duration and there were no follow-up sera collected from the patients enrolled in this study.

In preliminary analyses done on patients receiving this vaccine, no responses were detected against HPV16 E1, E2 or E6 (Kaufmann *et al.*, 2007). In contrast we found that certain immune responses to E6, E2, E1, and additionally to E4 have been developed and increased as more vaccine doses are given. An explanation for this unexpected response induction could be the nature of the vaccine. The vaccine itself may have a cytopathic effect on the cells and induce a cellular immune response causing the release of these early proteins and increase the chance of their exposure to the immune system. In the cells, viruses are still active in DNA transcription and replication. This event is important as it might indicate that administration of this vaccine could by induction of these early proteins share partly in stimulating the regression of the developed lesions due to the infection.

In conclusion, the results obtained in this study support that HPV16 L1/E7 cVLP vaccine has the properties of a prophylactic vaccine as it induce L1-binding, -neutralizing and additionally E7-specific antibodies. Induction of other early proteins might improve the suitability of the analyzed vaccine as a therapeutic vaccine to be given to already infected women.

Several preclinical studies used HPV16L1/E7 cVLP demonstrated the induction of E7-specific CTL that are able to inhibit tumor growth in mice (Greenstone *et al.*, 1998; Jochmus *et al.*, 1999; Schafer *et al.*, 1999). Therefore, more clinical studies to the cellular-immune response induced after vaccination with the cVLP should be conducted in order to give a clear and complete picture about this vaccine. If the suitability of cVLP to be used as prophylactic and at the same time as therapeutic vaccine has been proved in human, then these particles could be more advantageous over VLP used recently as only prophylactic vaccines and they may be applicable in women already infected.

## Figure legends:

### Figure 1

**Immune response of patients vaccinated with HPV16L1/E7 cVLP.** Each 12 CIN II/III patients were immunized with placebo or low dose (75µg), or high dose cVLP (250µg) at weeks 0, 2, 6, and 14. Blood samples for serology were taken at weeks 0, 2, 4, 8, 14, and 24. (A) Box plots of HPV16L1 reactivity in median fluorescence intensity (MFI) units. The dashed horizontal lines represent the seropositivity cut-off (150 MFI). (B) Correlation between MFI values at 1:100 dilution and endpoint dilution titers, defined as the highest serum dilution resulting in a signal above the cut-off (150 MFI). N on the x-axis represents the seronegative sera, titer<100 (C) Levels of HPV16L1 antibodies over time. The geometric mean titers and the 95% confidence intervals are shown throughout the 24 weeks of the study. Each line represents a different treatment group. Stars indicate the times of vaccination.

### Figure 2

#### **Vaccination with HPV16L1/E7 cVLP induces neutralizing antibodies.**

Sera collected from low and high doses vaccine recipients were analyzed for presence of neutralizing antibodies to HPV16 pseudovirions using the in vitro SEAP assay. Serum neutralization titers were also determined and were defined as the reciprocal of the highest dilution that caused at least a 70% reduction in SEAP activity. (A) Neutralization titers at W0 compared to W14. Dashed horizontal line represent the positivity cut-off (B) For each serum, neutralization titers were plotted against luminex binding antibody titers. N represents the group of seronegatives (titer<100, on x-axis) or the non-neutralizing sera (titer<60, on y-axis).

### Figure 3

#### **Vaccination with HPV16L1/E7 induces L1 cross-reactive antibodies.**

Study participants were investigated for the presence of L1 cross-reactive antibodies to different HPV types other than HPV16 (types grouped as alpha mucosal, alpha cutaneous and skin). (A) number of participants who seroconverted to any other HPV L1 within each group were counted in the different vaccine groups. Color codes are in the inserted legend. Cross-reactive antibodies were titrated and the geometric mean titers of the three different cross-reactive groups was calculated and compared to that of HPV 16 in (B) low-dose group and (C) the high-dose group. Stars indicated the times of vaccination.

#### **Figure 4**

##### **Vaccination with HPV16L1/E7 cVLP induces HPV16 E7 antibodies.**

Sera were investigated for the presence of E7 specific antibodies. (A) Box plots of HPV16E7 reactivity in median fluorescence intensity (MFI) units. The dashed horizontal lines represent the seropositivity cut-off (50 MFI). (B) Levels of HPV16E7 antibodies over time. The geometric mean titers and the 95% confidence intervals are shown throughout the 24 weeks of the study. Each line represents a different treatment group. Stars indicated the times of vaccination.

#### **Figure 5**

##### **Vaccination with HPV16L1/E7 induces E7 cross-reactive antibodies.**

Patients were investigated for the presence of E7 cross-reactive antibodies to different HPV types belonging to different alpha species (7, 9 and 10). (A) Number of participants who seroconverted to any other HPV E7 within each species was counted in the different vaccine groups. Color codes are in the inserted legend. Levels of E7 cross-reactive antibodies represented by the geometric mean of the MFI values were calculated and compared to that of HPV16 in (B) low-dose group and (C) the high-dose group. Stars indicated the times of giving the vaccine.

#### **Figure 6**

##### **Vaccination with HPV16L1/E7 induces other HPV 16 early proteins.**

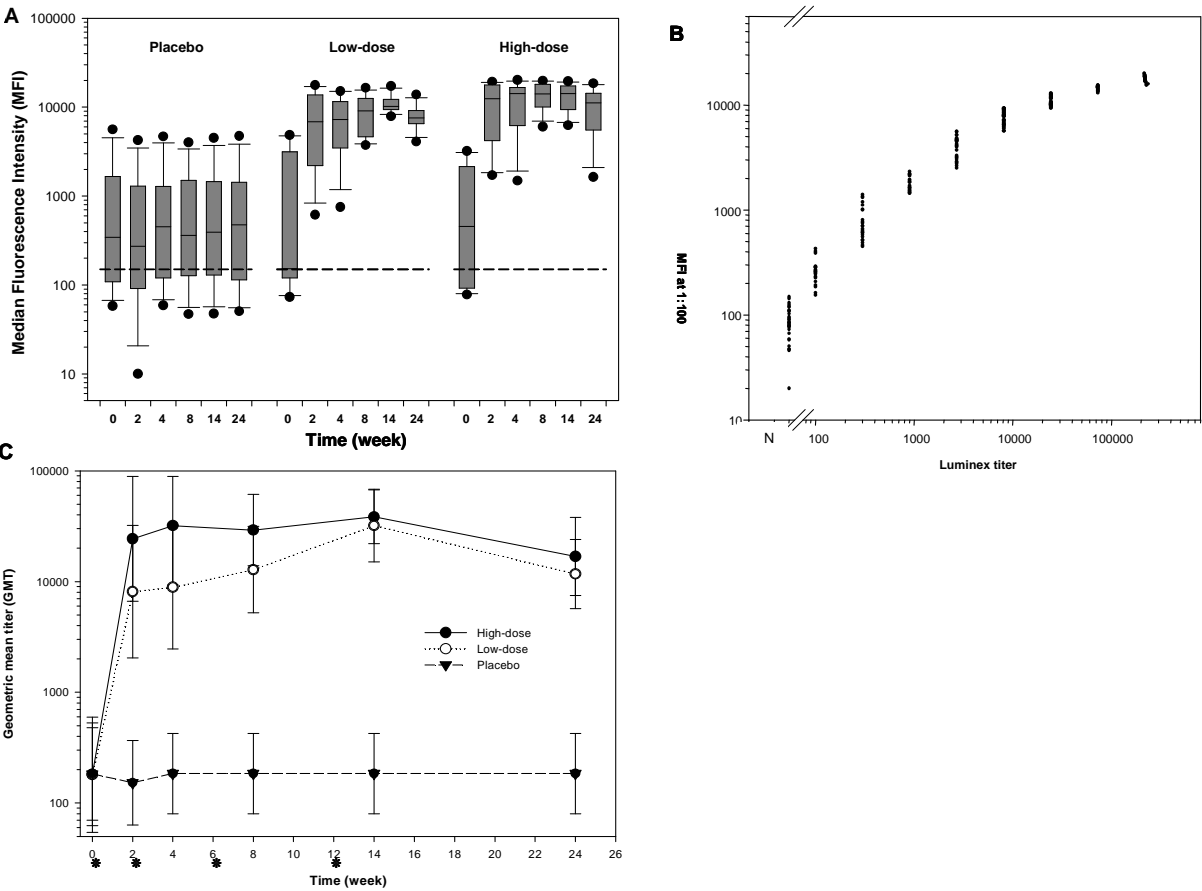
Sera were investigated for the presence of any immune response to other HPV16 early proteins including E6, E1, E2, and E4. Number of participants who developed increased immune response to any of these was counted among the three vaccine groups. E6 was the most frequent type, followed by E4, E1 and finally E2.

**Table 1** Summary of the immune responses to HPV16 L1 and E7 throughout 24 weeks after vaccination with HPV16L1/E7 cVLP

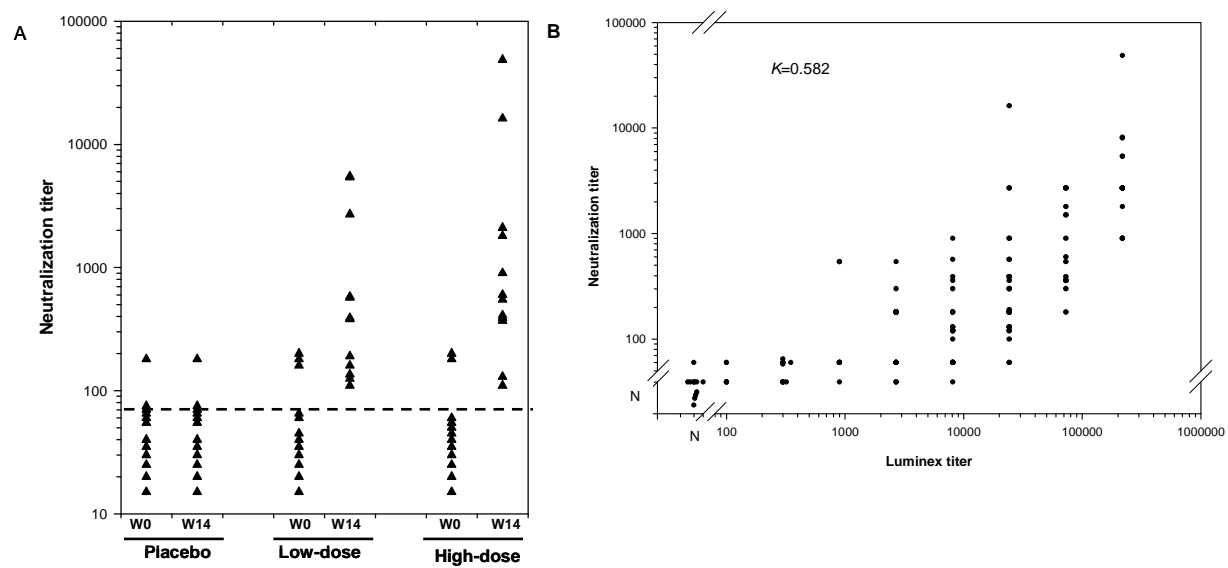
Antigen	week	Placebo				Low-dose				High-dose			
		Luminex median	range	Neutralization median	range	Luminex median	range	Neutralization median	range	Luminex median	range	Neutralization mediar	range
HPV16L1	W0	200	30-2700	40	20-180	65	30-2700	20	20-180	200	30-2700	20	20-180
	W2	100	30-2700			8100	300-218700	210	20-8100	72900	900-218700	720	60-2700
	W4	300	30-2700			8100	100-218700	110	20-2700	72900	900-218700	720	20-2700
	W8	200	30-2700			8100	2700-218700	180	20-2700	24300	2700-218700	240	60-1800
	W14	200	30-2700	40	20-180	24300	8100-218700	390	130-5400	24300	8100-218700	585	130-48600
	W24	100	30-900			8100	2700-218700	150	60-2700	16200	2700-218700	110	20-8100
HPV16E7 <sup>1</sup>	W0	13	1-370			15	6-137			27	8-636		
	W2	18	5-312			57	25-11062			793	39-2365		
	W4	12	6-492			108	14-4007			657	131-5820		
	W8	18	6-536			128	39-2562			557	186-5930		
	W14	15	4-212			115	13-2089			823	65-8207		
	W24	14	8-398			80	20-1602			435	29-7903		

<sup>1</sup> For HPV16E7, MFI values are shown

Rizk *et al*, Figure 1

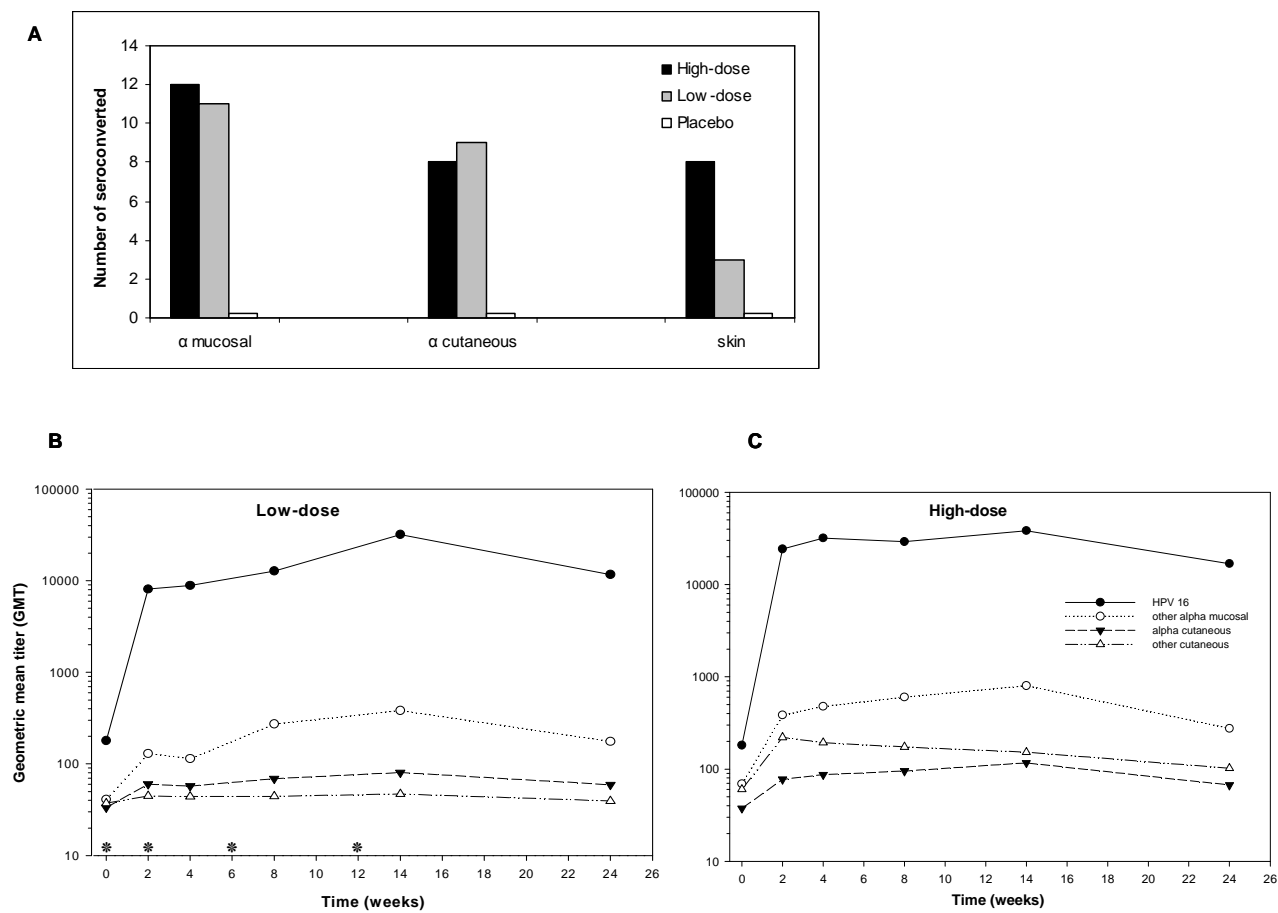


Rizk *et al*, Figure 2

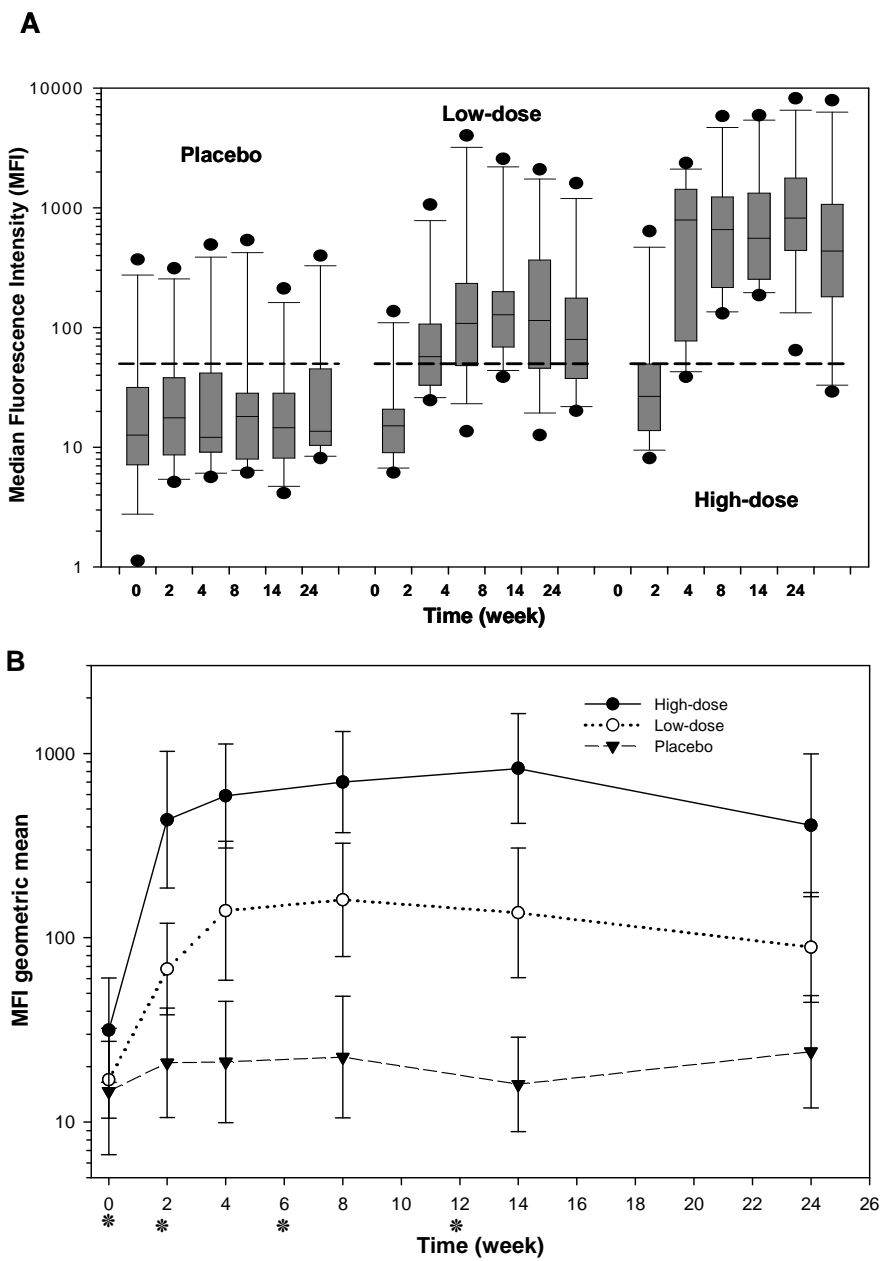




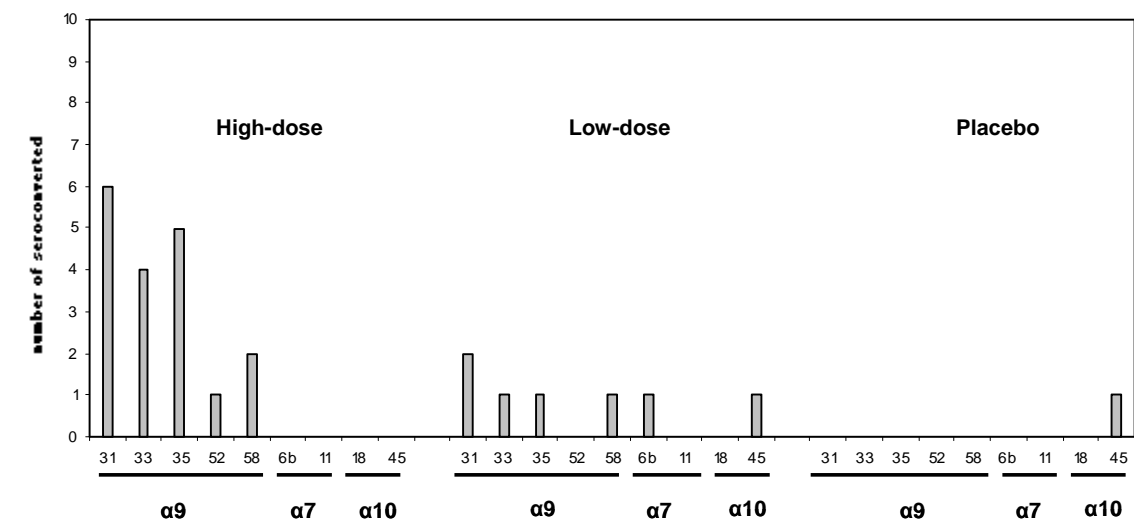
Rizk *et al*, Figure 3



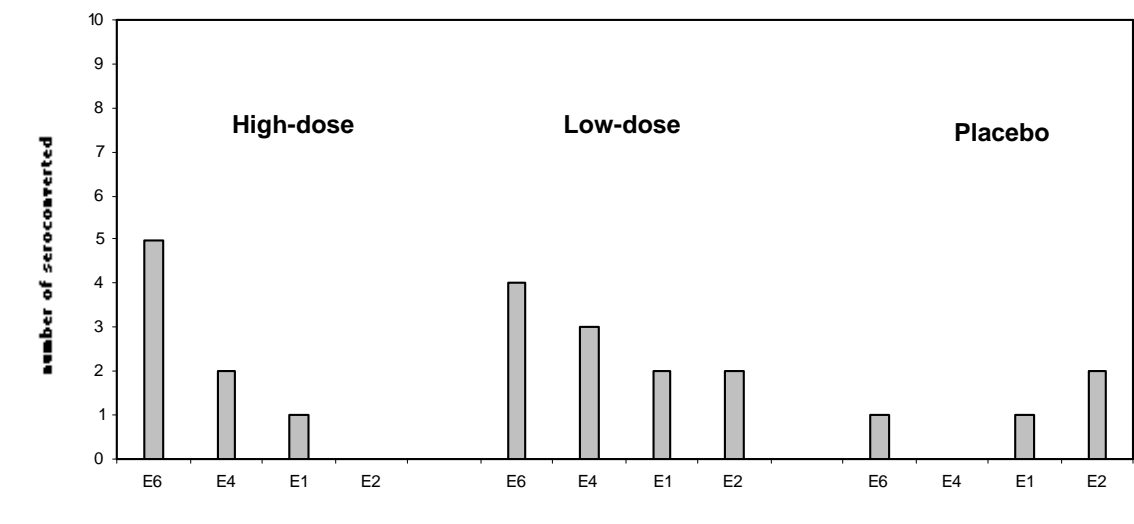
Rizk *et al*, Figure 4



Rizk *et al*, Figure 5



Rizk *et al*, Figure 6



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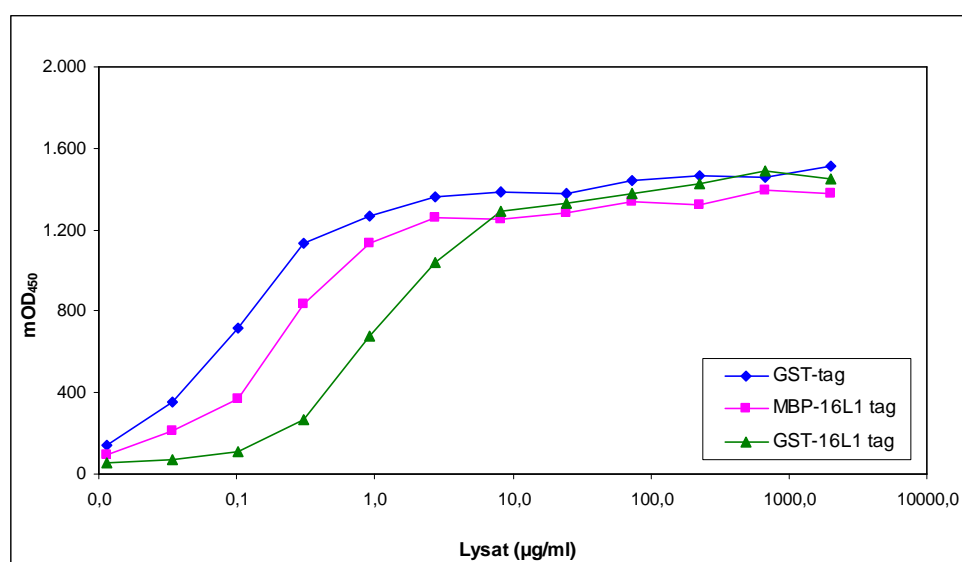


## **APPENDICES**

## Appendix 1

### Anti-tag ELISA

Quality and quantity of MBP-16L1tag fusion proteins were investigated using anti-tag ELISA. MBP-16L1tag obtained from *E.coli* strain *Rosetta* was titrated in a 1:3 dilution steps on a plate coated with 100  $\mu$ l/ml amylose and identified using mouse anti-tag monoclonal antibody. As a positive control for this experiment, GST-tag and GST-16L1tag fusion protein were titrated in parallel on a plate coated with 200 ng/ml glutathione-casein as capture protein. The antigen titration curves showed that MBP-16L1tag was expressed in full length and that its curve goes in parallel with those of GST-tag alone or GST-16L1tag fusion protein. MBP-16L1tag reached the saturation plateau earlier (at 3  $\mu$ g/ml) than MBP-16L1tag (at 8  $\mu$ l/ml).



**Figure 1. Titration curves of MBP16L1tag.** ELISA plates coated over night (4°C, 100 $\mu$ l/well) with either 100  $\mu$ g/ml amylose or 200 ng/ml glutathione casein were incubated with different concentrations of: MBP16L1tag expressed in *E.coli* Rosetta strain. GST-tag and GST16L1tag were titrated in parallel as a control. The bound protein to amylose was detected via the C-terminal tag epitope by mouse anti-tag monoclonal antibody. Absorbance values were expressed in milliunits (mA).

## Appendix 2

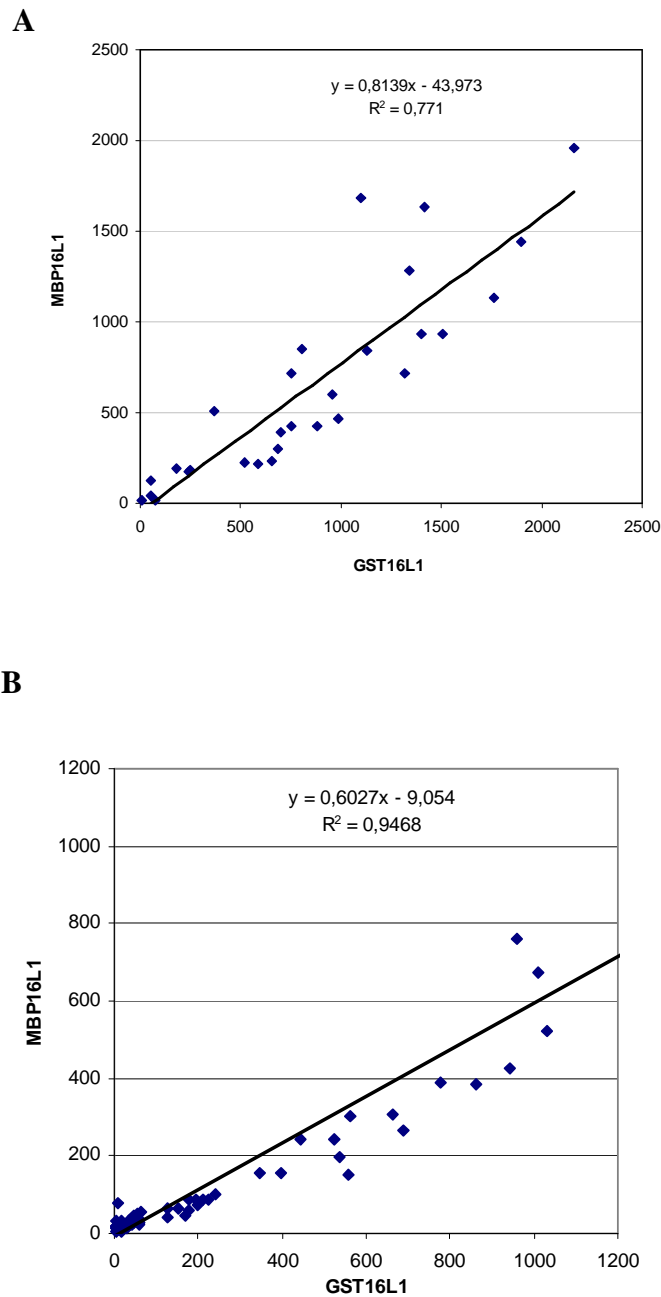
### Validation of MBP-16L1 using monoclonal antibodies

Quality of MBP-16L1tag as ELISA antigen was estimated by direct comparison with GST-16L1tag. For this aim, the reactivity of 29 previously defined monoclonal antibodies generated against HPV16 VLP was analyzed with MBP-16L1tag and GST-16L1tag in parallel. Absorbance value pairs obtained with the two ELISA systems for each monoclonal antibody were compared in *xy* plot (Figure 2A). Comparison showed agreement of the two result sets with linear regression  $R^2$  value of 0,77. A kappa value of 0,473 (95% CI range from -0,232 to 1,178) was found which indicated that the strength of agreement between the two ELISA formats is considered to be moderate.

### Validation of MBP-16L1 using human sera

In a second experiment to validate MBP-16L1tag as ELISA antigen, 72 sera from 36 patients clinically diagnosed with cervical intraepithelial neoplasia CIN (grade II or III) and enrolled in a Medigene HPV16L1/E7 cVLP vaccination study were analyzed by both GST-16L1tag and MBP-16L1tag ELISA. For each patient a pre- and a post-vaccination serum were analyzed. Comparison of the two ELISA data revealed a high correlation with  $R^2$  value of 0.95 and kappa value of 0.806 (95% CI range from 0,669 to 0,942) which indicated that the strength of agreement is considered to be very good (Figure 2B).

In summary, the validation experiments proved that MBP-16L1tag can react efficiently as well as GST-16L1tag does with HPV16L1 specific antibodies of different sources (mice and human). This indicated that fusing MBP to HPV16L1 had no effect in the folding of the protein and therefore on the presentation of the different epitopes.



**Figure 2. Validation of MBP16L1tag as ELISA antigens.** Reactivity of (A) HPV16L1 specific monoclonal antibodies generated against HPV16 VLP and (B) human sera from HPV16L1/E7 cVLP Medigene vaccination study determined by GST-16L1tag and MBP-16L1tag ELISA. For each monoclonal antibody or serum, result from GST-16L1tag ELISA is plotted on x-axis and that from MBP-16L1tag ELISA on y-axis. Values are given in mAU (milli absorbance unit). The linear regression line and the  $R^2$  value as measure of assay agreement are given.

## Appendix 3

### Titration of MBP-16L1tag blocking activity

After validation of MBP-16L1tag fusion protein as ELISA antigens, the next question was; at which concentration can the MBP-16L1tag block HPV 16 specific- and/or cross-reactive positive signal of a mAb and then of a human serum. To answer this question, the blocking activity of the MBP fusion protein was titrated against type-specific and cross-reactive mAb and also against different human sera with different reactivity patterns.

### Titration of MBP-16L1tag blocking activity using monoclonal antibodies

To determine the appropriate MBP-16L1 concentration that could be used to block HPV16 specific and cross-reactive epitopes, MBP-16L1 fusion protein blocking activity was titrated using different HPV 16 mono-specific and cross-reactive monoclonal antibodies. The following antibodies were used in these experiments:

<b>H16.5A</b>	<i>strongly reactive with HPV16 but not HPV18</i>
<b>H16.9A</b>	<i>strongly reactive with HPV16 and weakly with HPV18</i>
<b>H16.H5</b>	<i>strongly reactive with HPV16 and intermediate with HPV18</i>
<b>H16.8B</b>	<i>strongly reactive with both HPV16 and 18</i>

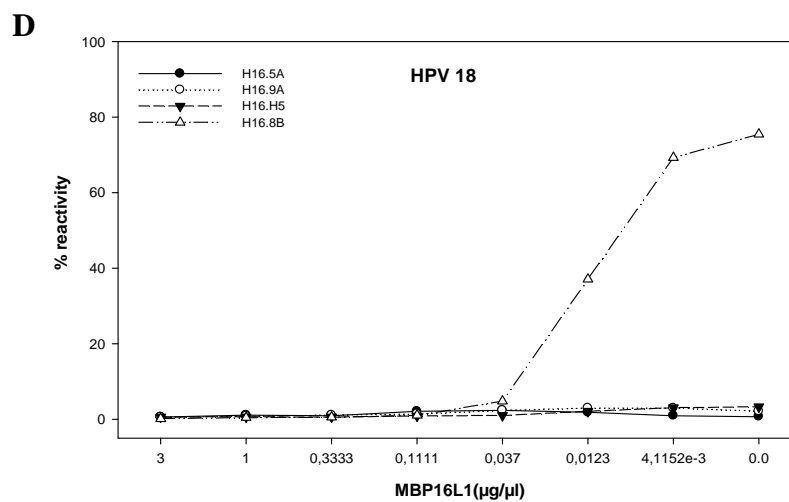
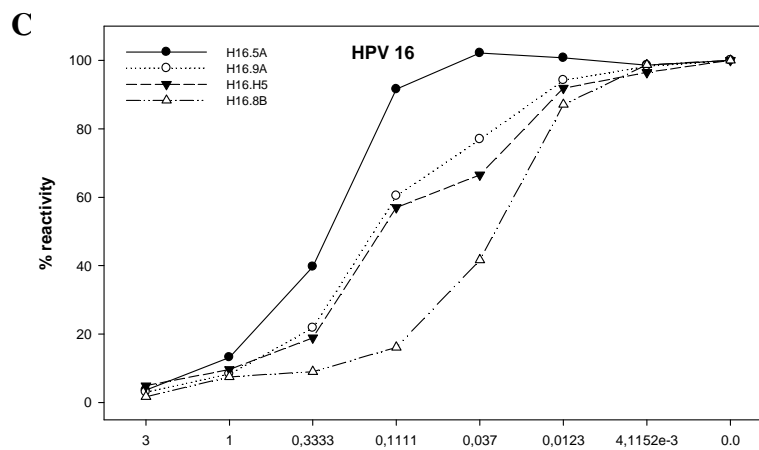
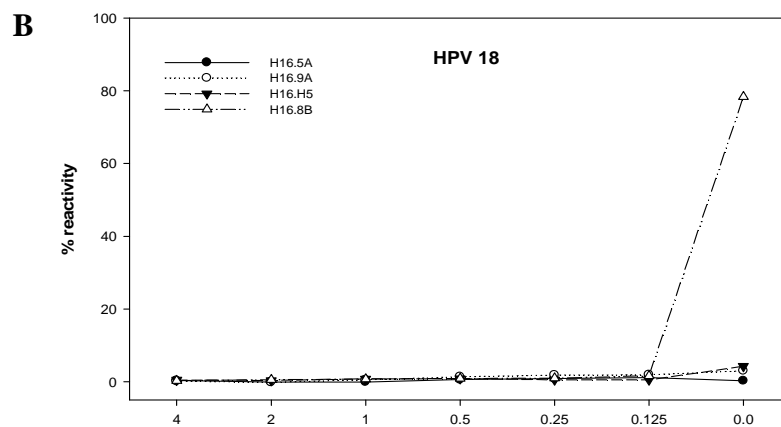
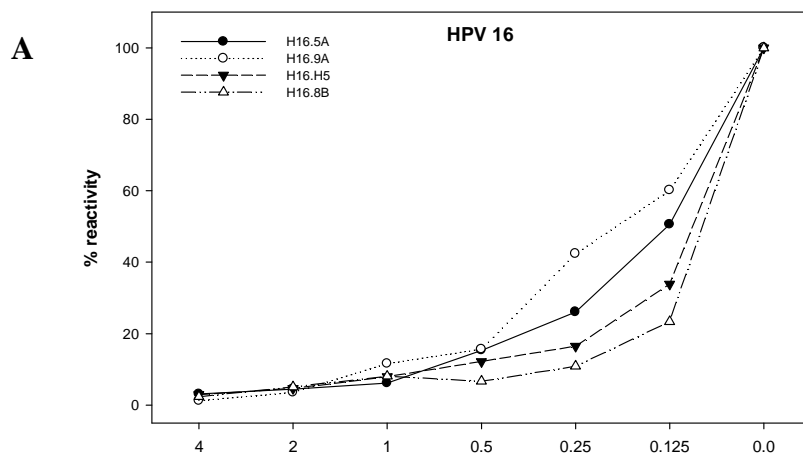
In the first experiment, the mAb all diluted 1:2500 were preincubated with different concentrations of MBP-16L1 starting from 4 µg/µl and diluted in 1:2 steps down to 0,0 µg/µl. GST-tag was always used as a negative control.

As expected after the preabsorption step, all of the four monoclonal showed different degrees of inhibition in their reactivity when examined further with GST-16L1. The reactivity of *H16.8B* and *H16.H5* was reduced by ≥70% when preabsorbed with 0,125µ/µl MBP-16L1. *H16.5A* showed 70% reduction in reactivity at MBP-16L1tag concentration of 0,25 µg/µl. The least monoclonal antibody to loose binding activity to GST-16L1 was *H16.9A* which achieved 70% reduction in its activity when preabsorbed with 0,5 µg/µl MBP-16L1 (Figure 3A). The same mAb were further examined with GST-18L1 in order to ensure the specificity of the assay and also to investigate if the cross-reactive epitopes recognized by three of these mAb can be also blocked by MBP-16L1 preabsorption step. The specificity was achieved as in absence of MBP-16L1, *H16.5A* did not react with GST-18L1, whereas *H16.8B* reacted

very strongly and *H16.9A* and *H16.H5* reacted very weakly as expected. After preabsorption with MBP-16L1, more than 90% blocking in the reactivity of the later three mAb was achieved at a concentration of 0,125µg/µl (Figure 3B).

The common observation in this experiment was the fast and direct decrease in the reactivity of the mAb at the lowest MBP-16L1 concentration used for the preabsorption (0,125µg/µl). However, we were interesting in investigating how would be the decrease in reactivity if using smaller concentrations than 0,125µg/µl; therefore a second experiment was performed using lower concentrations of MBP-16L1. The same mAb used in the first experiment were preincubated with different concentrations of MBP-16L1 starting from 3 µg/µl and diluted in 1:3 steps down to 0,0 µg/µl. A nice sigmoidal reactivity titration curves were obtained. Up to an MPB-16L1 concentration of 0,1µg/µl retained *H16.5A* most of its binding activity ( $\geq 90\%$ ). The other cross-reactive mAb *H16.9A*, *H16.H5* and *H16.8B* hold  $\geq 80\%$  of their reactivity only up to 0,01µg/µl of MBP-16L1; they lost their binding activity faster than the mono-specific *H16.5A*. The data showed also that at MBP-16L1 concentration of at least 1µg/µl, all the specific and cross-reactive epitopes can be blocked, however, blocking of cross-reactive epitopes can be achieved using less amount of the MBP-16L1 (Figure 3C). When testing the mAb with GST-18L1, again only the strongly cross-reactive *H16.B8* showed a significant signal. In comparison to the binding activity to HPV16, most of the reactivity ( $>90\%$ ) to HPV18 (cross-reactive type) inhibited by using at least 0,03µg/µl (Figure 3D) which is 27 folds lower than the amount necessary to block reactivity to the specific HPV type.

From theses experiments we concluded that using at least 1µg/µl is sufficient to block both reactivity to the type-specific as well as the cross-reactive epitopes.



**Figure 3 (continued). Titration of MBP16L1 blocking activity using monoclonal antibodies.** Four mAb were preincubated with MBP-16L1 concentrations from 4 µg/µl titrated down to 0.0 µg/µl in 1:2 steps and tested for their reactivity to (A) HPV 16L1 and (B) to HPV 18L1. In a second experiment, the same mAb were preincubated with MBP-16L1 concentrations from 3 µg/µl titrated down to 0.0 µg/µl in 1:3 steps and again tested for their reactivity to (C) HPV 16L1 and (D) HPV 18L1. Data showed that at least 1µg/µl MBP-16L1 is sufficient to block both HPV-16 type-specific and cross-reactive epitopes.

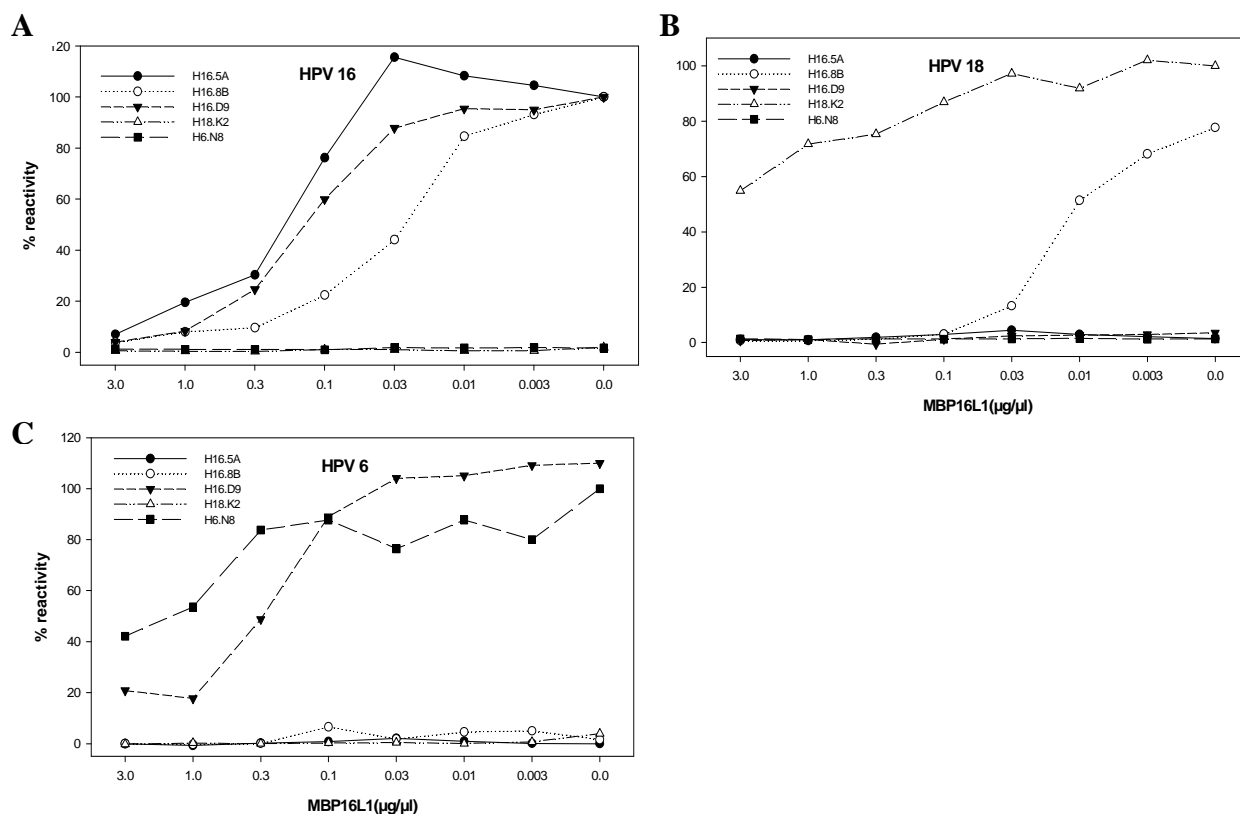
### **Specificity of MBP16L1 blocking activity**

Using the mAb, we were able to investigate whether the blocking activity of MBP-16L1 was specific or not; whether only HPV 16 specific and cross-reactive epitopes are blocked and not other epitopes specific for other HPV types. To this end, the following mAb were preincubated with different concentrations of MBP-16L1 as described in the later section:

<b>H16.5A</b>	<i>strongly reactive only with HPV16</i>
<b>H16.8B</b>	<i>strongly reactive with both HPV16 and HPV18</i>
<b>H16.D9</b>	<i>strongly reactive with both HPV16 and HPV6</i>
<b>H18.K2</b>	<i>strongly reactive only with HPV18</i>
<b>H6.N8</b>	<i>strongly reactive only with HPV6</i>

After preabsorption, the different mAb were examined with GST-L1 fusion proteins of HPV 16, 18 and 6. As expected neither *H18.K2* nor *H6.N8* reacted with HPV16 (Figure 4A) but only with HPV18 (Figure 4B) and 6 respectively (Figure 4C). Furthermore, these two mAb had not been affected with the preabsorption process with MBP-16L1 and their reactivity to the specific HPV type remain more or less stable up to an MBP-16L1 concentration of 1µg/µl; however, preincubation with higher concentrations may also decrease the binding activity even to the specific HPV type. As earlier concluded from the previous blocking experiments, the data confirms again that HPV16 cross-reactive antibodies lose their reactivity faster than the type-specific ones and at 1 µg/µl, they all lost ≥80% of their binding activity (Figure 4).





**Figure 4. Determination of specificity of MBP-16L1 blocking activity.** Three HPV 16, one HPV18 and one HPV 6 specific mAb were preincubated with different MBP-16L1 concentrations from 3 μg/μl titrated down to 0.0 μg/μl in 1:3 steps and tested for their specific reactivity to (A) HPV 16L1 (B) HPV 18L1 and (C) HPV 6L1. None of HPV 18 or 6 type-specific mAb was blocked.

## Appendix 4

### Titration of MBP-16L1tag blocking activity using human sera

Since the main objective of this project is to differentiate the specific from cross-reactive signals in serological studies, it was important to check if similar results concerning the blocking activity of MBP-16L1 could be obtained when use selected human sera instead of the experimentally produced mAb. We wanted by using human sera to titrate the blocking activity of MBP-16L1 and determine the appropriate concentration that could achieve almost complete inhibition of HPV 16 specific and cross-reactive epitopes recognized by a serum.

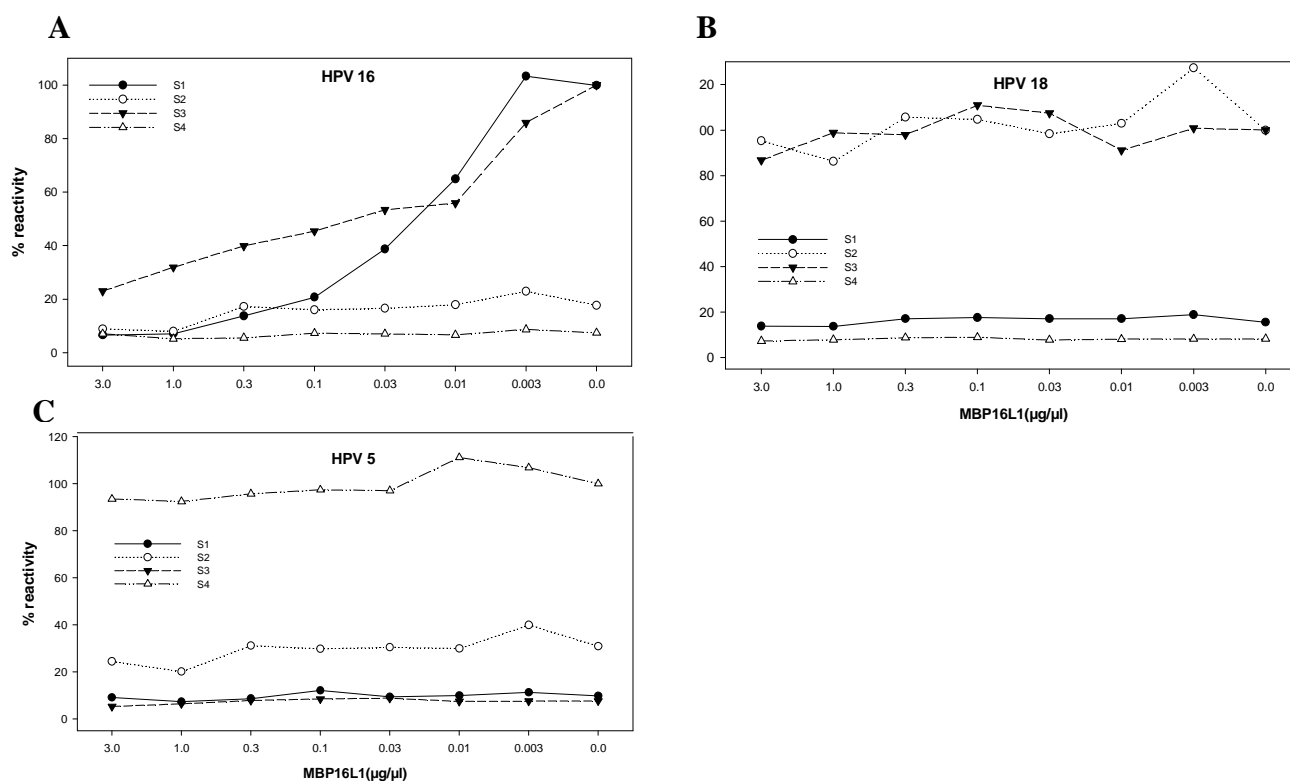
Three sera from Madras-Algiers study and one from Rom case-control study were chosen for these experiments and they were tested in duplicates:

<b>Madras-Algiers</b>	Serum 1 ( <b>326</b> )	<i>strongly reactive with only HPV16</i>
	Serum 2 ( <b>603</b> )	<i>strongly reactive with only HPV 18</i>
	Serum 3 ( <b>523</b> )	<i>strongly reactive with both HPV 16 and 18</i>
<b>Rom (case-control)</b>	Serum 4 ( <b>20210</b> )	<i>strongly reactive with only HPV 5</i>

The sera were preincubated as previously described with different concentrations of MBP-16L1 and then their reactivity to GST-L1 of HPV 16, 18 and 5 was tested in ELISA.

The HPV16 assay showed that both S3 and S4, as expected, did not react and the recorded activity was almost less than 20%. More than 80% of S1 reactivity was blocked by preabsorption with 0,1µg/µl MBP-16L1, in contrast, S2 which in previous investigations done by other colleagues was strongly reactive with both HPV16 and 18 lost 80% of its reactivity

Therefore, and to ensure complete blocking, 2 µg/µl MBP-16L1 will be applied in the following experiments.



**Figure 5. Titration of MBP-16L1 blocking activity using human sera.** Four human sera with different reactivity patterns were preincubated with different MBP-16L1 concentrations from 3 µg/µl titrated down to 0.0 µg/µl in 1:3 steps and tested for their reactivity to (A) HPV16L1 (B) HPV18L1 and HPV 5L1. None of HPV 18 or HPV 5 type-specific sera was blocked.

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December 05 DKFZ-Annual Poster presentation, Heidelberg, Germany.

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## Publications

Rizk R., Christensen N. D., Michael K., Müller M., Sehr P., Pawlita M. (2007) Reactivity Patterns of 92 monoclonal antibodies with 15 Human papillomavirus types. *Manuscript accepted for publication in Journal of General Virology*.

Kjaerheim K., Roe O., Waterboer T., Sehr P., Rizk R., Dai H., Sandeck H., Larsson E., Anderson A. and Pawlita M. (2006) No evidence of an association of SV40 antibodies or DNA fragments in pre-diagnostic serum samples with mesothelioma. *Int J Cancer*. 120, 2459-2465.

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Hiermit erkläre ich an Eidesstatt , dass ich die vorliegende Arbeit selbständig und ohne unerlaubte Hilfsmittel durchgeführt habe.

Heidelberg,

9/11/2007

Raeda Z. Rizk