29

A STUDY OF THE IMMUNE RESPONSE TO HUMAN PAPILLOMAVIRUS TYPES CAUSING CERVICAL CANCER

Dianne Jean Marais

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This thesis is dedicated to my husband, Ian and my children,
Philip, Jeanne and Sarshen.

"I left science for you all, but I returned to find it again."

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CONTENTS

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GLOSSARY

β-gal	β-galactosidase
aa	Amino acid
AIN	Anal intraepithelial neoplasia
BCG	Bacille Calmette-Guerin
BPV	Bovine papillomavirus
BSA	Bovine serum albumin
BUdR	5-bromodeoxyuridine
CaCx	Cervical cancer
CAM	Chorio-allantoic membrane
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
CIS	Carcinoma in situ
CM	Cervical mucus
CMI	Cell mediate immunity
CMIS	Common mucosal immune system
CO_2	Carbon dioxide
COPV	Canine oral papillomavirus
CRPV	Cottontail rabbit papillomavirus
CTB	Cholera toxin B
CTL	Cytotoxic T lymphocyte
CV-1	African green monkey kidney cells
CVL	Cervico-vaginal lavage
DMEM	Dulbeccos minimum essential medium
DMSO	Di-methyl sulphoxide
DNA	Deoxyribonucleic acid
dOD	Difference in optical density
DOH	Department of health
DTH	Delayed-type hypersensitivity

ELISA	Enzyme-linked immunosorbent assay
HBS	Hepes-buffered saline
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HPV	Human papillomavirus
HSIL	High grade squamous intraepithelial lesion
HSV	Herpes simplex virus
IARC	International Agency for Research on Cancer
ICE	Insect cell extract
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
i.p.	intraperitoneal
kDa	Kilo Dalton
LSIL	Low grade squamous intraepithelial lesion
Mab	Monoclonal antibody
MHC	Major histocompatibility complex
MI	McIllvains
min	Minute
NIH	National Institute of Health
NP	Nucleoprotein
OD	Optical density
OR	Odds ratio
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pfu	Plaque forming unit
PV	Papillomavirus
rBCG	Recombinant BCG
RIPA	Radioimmunoprecipitation assay

RNA	Ribodeoxynucleic acid
RR	Relative risk
rVv	Recombinant vaccinia virus
SC	Secretory component
SCID	Severe combined immunodeficiency
SD	Standard deviation
SEM	Standard error of the mean
S-IgA	Secretory IgA
STD	Sexually transmitted disease
Th	T-helper cell
TK	Thymidine kinase
TK^+	Thymidine kinase gene expressed
TK^-	Thymidine kinase gene not expressed
VLP	Virus-like particle
VSV	Vesicular stomatitis virus
Vv	Vaccinia virus
WHO	World Health Organisation
WR	Western Reserve
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactosidase

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4.

Chapter 5.

Chapter 6.

Chapter 7

LIST OF TABLES

Chapter 1

Chapter 2

Chapter 3

Chapter 4.

Chapter 5.

Chapter 6

Chapter 7

ABSTRACT

This study constitutes the first reported assessment in Africa of the antibody responses to human papillomavirus (HPV) proteins and the association of these responses with cervical disease. Immunoglobulin G (IgG) antibodies to HPV antigens were measured by enzyme-linked immunosorbent assay (ELISA) in the serum of children, female blood donors, and patients with cervical intraepithelial neoplasia (CIN). A significant association with CIN was found for IgG antibodies to an E2 peptide (E2-16, $P = 0.039$) and HPV-16 virus-like particles (VLP-16, $P = 0.002$). This association was found to be age-dependant in women with CIN, with a higher antibody seropositivity rate to E2-16 noted in women over 40 years and a lower antibody seropositivity rate to VLP-16 found in the older women. This implied that in women with CIN, VLP-16 antibodies were markers of CIN in younger women and E2-16 antibodies markers of CIN in older women. A high antibody seroprevalence rate was noted in the children's sera to E2-16 (44.5%), with a decreased seroprevalence in older children.

The assessment of seroresponses to VLPs of HPV types 16, 18, 31, 33 and 45 in the women with cervical cancer (CaCx) as well as women with CIN, blood donors, San people and children is the first study of antibody responses to 5 oncogenic HPV VLP types. The prevalence rate to the 5 HPV VLP types was lowest amongst the children but 27.3% of Cape Town children and 22.6% of the San children were seropositive to at least one VLP type, as were 58% of all adult women tested. The San adults displayed high seroprevalence rates to VLP-18 and VLP-45, corroborating other evidence of the high prevalence rates of HPV-18 and HPV-45 in parts of Africa. The antibody prevalence rates among the CIN patients' sera were elevated above that of blood donors, for all VLP types. VLP antibody prevalence rates in the CaCx patient sera were higher than prevalence rates of the CIN patients for all VLP types, with the exception of antibodies to VLP-16. Association with disease was found for IgG antibodies to VLP-16 and VLP-45 in women with CIN, compared with blood donor controls. This is the first report of an association of antibodies to VLP-45 with cervical disease. Seroprevalence to multiple VLP types was evident in all the groups studied, especially so in the women with cervical disease and was most common in older women with CaCx (55%).

The antibody responses appeared type–specific, but some cross reactivity was apparent, although not confirmed. Cross reactivity between antibodies to VLP-16 with antibodies to VLP-31, as well as antibodies to VLP-18 with VLP-45 antibodies appeared likely in some women.

Anti-VLP-16 IgA antibody levels in oral fluid of women with CIN and CaCx were compared with those of normal healthy control individuals. Prevalence rates were 55.7% compared with 8% in the controls ($P = 0.000002$). A group of 26 women with CIN 2 or CIN 3 (CIN2/3) were examined for IgA and IgG antibodies to VLP-16 in serum and cervical mucus as well as oral fluid. The percentage of women with VLP-16 antibodies was highest for serum IgA (65.4%), with 61.5% of these women having detectable serum IgG and 42% and 38.5% IgA and IgG antibodies, respectively in cervical mucus. The serum IgG responses correlated best with the presence of HPV-16 deoxyribonucleic acid (DNA) at the cervix, with an 87.5% IgG antibody prevalence in women with CIN2/3 who were HPV-16 DNA positive.

Commercial sex workers, at high risk of cervical disease, were examined for their HPV DNA presence and cervico-vaginal lavage and serum samples from these women were examined for anti-VLP-16 antibodies. Many of these women were infected with human immunodeficiency virus (HIV-positive) and others were not infected with HIV (HIV-negative). The HIV-positive women displayed a significant increase in the prevalence of HPV DNA (85%) compared with HIV-negative women (42%, $P = 0.00001$) and a significant increase in cervical anti-VLP-16 IgG antibodies ($P = 0.002$) and a significant decrease in anti-VLP-16 serum IgA ($P = 0.012$) compared with HIV-negative women. This was the first published report of cervical antibodies to VLP-16 in HIV-positive women.

A novel recombinant vaccinia virus (rVv)-HPV-16 L1 challenge model was developed to evaluate the efficacy of the cell mediated immune response following HPV-16 VLP immunisation in mice. Intra-peritoneal vaccination of BALB/c mice with HPV-16 VLPs afforded protection against viral challenge from rVv expressing HPV 16 L1 ($VvL1_R$-16). Protection was demonstrated by a $4.6\log_{10}$ reduction in $Vv\text{-}L1_R$-16 ovarian titres in vaccinated BALB/c mice, compared with unvaccinated mice. The inability to infect laboratory animals

with HPV has in the past abrogated the testing of HPV vaccines in animal systems. Mice immunised intragastrically with VLP-16 were also protected from $VvL1_R$-16 challenge. Three rBCG constructs expressing different lengths of the L1 protein of HPV-16 were also tested for their ability to protect mice from $VVL1_R$-16 challenge, and all induced $>4\log_{10}$ levels of protection. The mouse challenge model demonstrated a quantitative measure of the protection induced by potential HPV vaccines.

PUBLICATIONS

The following publications have resulted from the work included in this thesis:

1. Marais DJ, Rose RC, Williamson A-L (1997): Age distribution of antibodies to human papillomavirus in children, women with cervical intraepithelial neoplasia and blood donors from South Africa. Journal of Medical Virology 51: 126-131.

2. Marais DJ, Passmore J-A, Maclean J, Rose RC, Williamson A-L (1999): A recombinant human papillomavirus 16 L1-vaccinia virus murine challenge model demonstrates cell-mediated immunity against HPV virus-like particles. Journal of General Virology 80: 2471-2475.

3. Marais DJ, Rose RC, Lane C, Aspinall S, Bos P, Williamson AL (2000): Seroresponses to virus-like particles of human papillomavirus types 16, 18, 31, 33 and 45 in San people of southern Africa. Journal of Medical Virology 60: 331-336.

4. Marais DJ, Rose RC, Lane C, Kay P, Nevin J, Denny L, Soeters R, Dehaeck CMC, Williamson AL (2000): Seroresponses to human papillomavirus types 16, 18, 31, 33 and 45 virus-like particles in South African women with cervical cancer and cervical intraepithelial neoplasia. Journal of Medical Virology 60: 403-410.

CHAPTER 1. INTRODUCTION

The insidious nature of human papillomaviruses (HPVs) makes them highly successful viruses and coupled with their ability to remain covertly latent for years, certain types have oncogenic potential. These human papillomavirus types are the causative agents in cancer of the uterine cervix, the second most common malignancy in women worldwide. Half a million women are diagnosed yearly with cervical cancer and 50% of these women die of the disease [Pisani *et al.*, 1993].

1.1. Human papillomavirus (HPV) and cancer

In 1995 the World Health Organisation (WHO) officially declared human papillomavirus (HPV) a human carcinogen [IARC/WHO, 1995]. This declaration followed extensive molecular, clinical and epidemiological work with human patients and animal models worldwide. All of which was subsequent to the confirmation of the viral aetiology of genital warts by Dunn and Ogilvie [1968] and the suggestion in the mid-1970s by zur Hausen [1977] that papillomaviruses are associated with genital cancer. The amassed evidence implicated HPV as the causative agent in squamous cell cervical carcinomas and their precursors [Richart *et al.*, 1998]. The association of specific HPV types with cervical cancer (CaCx) is strong with odds-ratios of >15 in case control studies and is consistent in countries where the risk of CaCx is either high or low [Bosch *et al.*, 1995]. Evidence is that HPV infection precedes the development of cervical intraepithelial neoplasia (CIN) stages 2 and 3 (CIN2/3). This is biologically plausible with the evidence that the proteins of the genes of HPV type 16 (HPV-16) and HPV-18 E6 and E7, have transforming potential [Halbert *et al.*, 1991; Munger *et al.*, 1989]. The oncogenic types are consistently found in over 90% of CaCx [Bosch *et al.*, 1995] or possibly all CaCx [Walboomers *et al.*, 1999]. The WHO declaration was followed in 1996 by the National Institute of Health (NIH) Consens Statement. Here experts prepared abstracts from all the available literature and these were presented to a panel. The panel resolved conflicting recommendations and presented their conclusions. " Carcinoma of the cervix is causally related to infection with human papillomavirus (HPV). Reducing the rate of HPV infection by changes in sexual behaviors in young people and/or through the development of an effective HPV vaccine would reduce the incidence of this disease....."

1.2. Papillomavirus (PV) structure and genome organisation

The human papillomaviruses (HPVs), which cause cervical cancer, are classified in one of two subfamilies of a large family of deoxyribonucleic acid (DNA) viruses known as *Papovaviridae.* Contained in the second subfamily are the Polyoma viruses [Howley, 1996]. All these viruses reside in the nucleus and all are tumor viruses, whose oncogenic proteins act by complexing with host regulatory proteins to disrupt the cellular mitotic and DNA repair processes. PVs are very widely distributed, incurring infections and neoplasms in many mammalian and avian species. Cross-infection between species does not occur except possibly between cows and horses [Richart *et al.*, 1998].

The whole PV virion is the infectious unit and consists of a DNA core surrounded by a protein capsid. The virions are non-enveloped with 72 capsomeres which encapsidate the genome and are approximately 45-55nm in diameter [Baker *et al.*, 1991]. The capsomeres are arranged on a T=7 icosahedral lattice (Fig 1.1). The capsid contains the major structural protein L1 (55kDa) and the minor L2 protein (74kDa) with 5-10 times more L1 than L2 [Orth *et al.*, 1978].

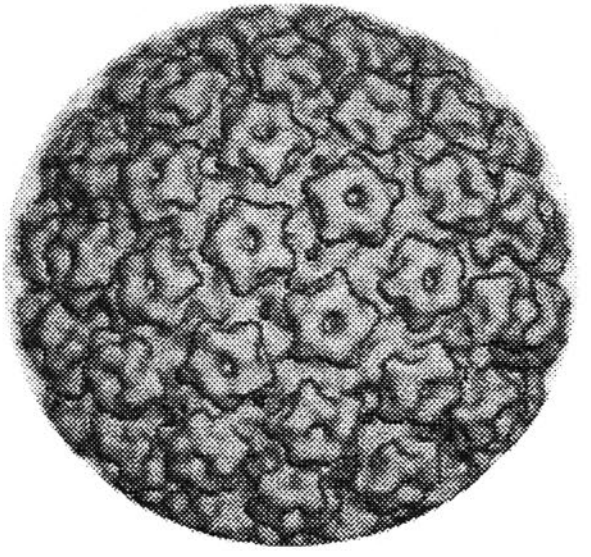

Fig. 1.1. A 3-D image of a cryoelectronmicrograph of an HPV-1 L1 capsid. (from Hagensee *et al.* [1994], with permission).

The tightly coiled, circular double-stranded DNA genome of PVs contains 8000 base pairs and encodes at least 8 proteins [Pfister and Fuchs, 1987]. The HPV genome can be divided into three regions [Seedorf *et al.*, 1985]: an early region that encodes early proteins (E1

through E7) that function in the regulation of protein transcription, the replication of viral DNA (E1 and E2), and cell transformation (E5, E6 and E7) (Fig 1.2.). These proteins interact with the host genome and programme the host cell to produce viral DNA. There is a late region that is activated after viral DNA replication that encodes late expressed proteins, which are the structural proteins of the viral capsid (L1 and L2). These surround the DNA to complete the infectious unit, the virion [Frizlaff *et al.*, 1988]. The L1 proteins are highly conserved and similar in all HPV types, but the L2 or minor protein is very variable. This L2 variability probably accounts for much of the antigenic differences between HPV types [Richart *et al.*, 1998]. The E4 gene although located in the early region, is translated late and the E4 protein is involved in productive infection, possibly assisting in the viral release from the cell [Roberts *et al.*, 1993]. A control region, the viral long control region, or upstream regulatory region is the noncoding region of the virus and contains replication and transcriptional control elements including the only known promoter [Shah and Howley, 1996] (Fig 1.2). The major function of the upstream regulatory region is the control of viral replication.

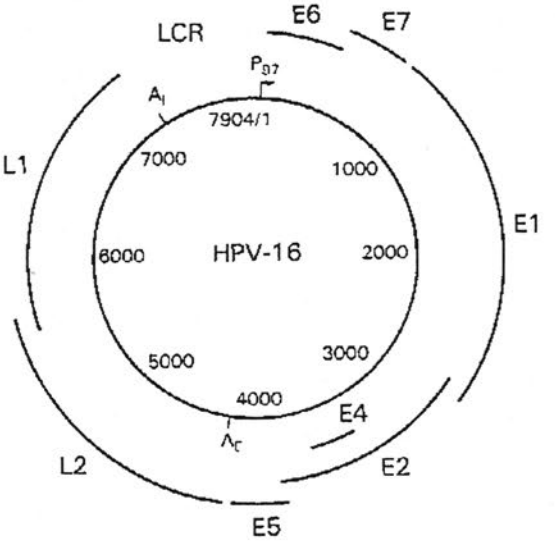

Fig.1.2. HPV-16 genomic map. Early region genes are designated E1 through E7 and L1 and L2 are the late region genes. The LCR is the long control region, P_{97} is the transcriptional promoter and A_E and A_L, early and late polyadenylation sites.

There are presently over 80 types of HPV (with new types being described regularly) which all reveal a well-conserved general organisation [Volter *et al.*, 1996]. Classification is based on the host species that they infect and the degree of DNA sequence homology within specific viral genes [Delius and Hofman, 1994]. New HPV types must share less

than 90% homology within the L1 gene and early genes, compared with existing types [Beutner and Tyring, 1997]. There is complete sequence data on almost all the known HPV types [Myers *et al.*, 1996] and a phylogenetic tree based on the L1 amino acid sequences is shown in Fig. 1.3.

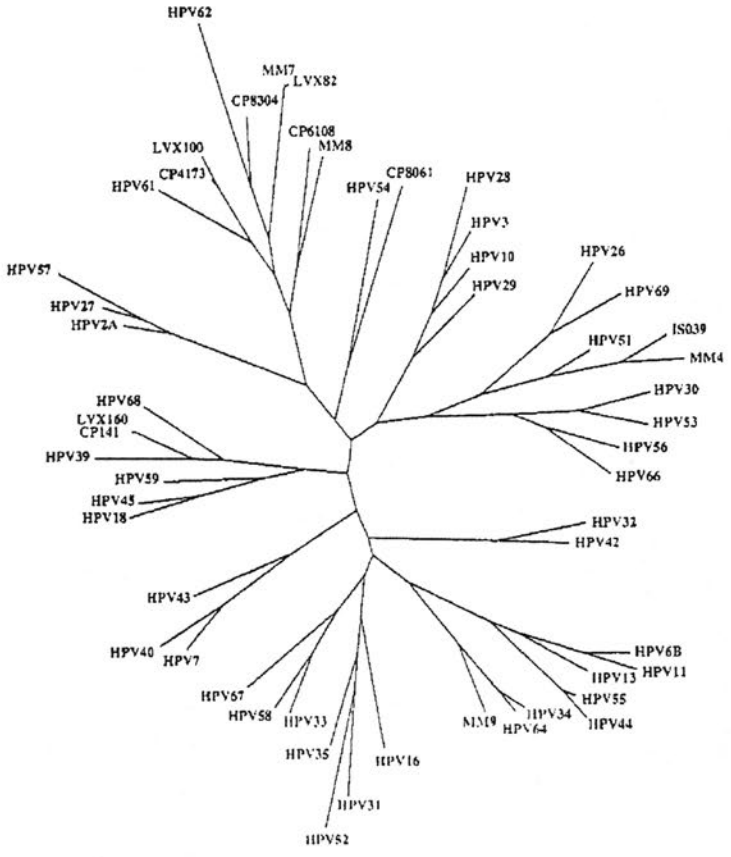

Fig.1.3. The phylogenetic tree of HPV types based on the L1 amino acid sequence [Myers *et al.*, 1994, with permission].

1.3. HPV replication and malignant transformation.

The human papillomaviruses fall into two main groups, those infecting cutaneous epithelium and those infecting mucosal epithelium (mucosotropic), and there also exists a mucocutaneous infection seen in dual infection of skin and oral mucosa. PVs have a specific tropism for squamous epithelial cells [Howley, 1996] and fundamentally induce proliferation of the cells that they infect, producing warts (benign tumors). Certain oncogenic HPV types will transform cells and this could result in malignancy [Reid *et al.*, 1987], which is a multistep process involving the accumulation of cellular genetic changes [Richart *et al.*, 1998]. Infection is of basal cells in the epithelium (keratinocytes) as PV must infect a cell capable of dividing, possibly at an area of trauma. The L1 protein binds

to the cell surface [Roden *et al.*, 1994] to a highly conserved PV-specific receptor in the cell membrane [Sapp *et al.*, 1996]. There is limited knowledge about the uptake and endocytosis of the virus and the uncoating of the viral DNA. Protein expression is regulated by the differentiation status of the keratinocytes [Stoller *et al.*, 1992] which HPV has infected at the cervix. The basal layers of the epithelium support low expression of the proteins E1 and E2 which occurs in the nucleus [Crum *et al.*, 1986]. In the suprabasal layers of the epithelium E6 and E7 proteins appear, also present in small amounts [Greenfield *et al.*, 1991]. As differentiating cells move toward the more superficial cell layers, the E4 protein and viral capsid antigens L1 and L2 are produced and viral assembly takes place. These proteins are only produced in cells undergoing terminal maturation and their production is thought to be regulated by the fact that the codon composition of the L1 and L2 genes is different compared with most mammalian genes [Zhou *et al.*, 1999]. The matching of the tRNA availability to the rare codon usage may be the factor restricting PV late gene expression to differentiated epithelium. The number of viral particles increases as the differentiating cells mature and the DNA is wrapped in a protein coat only in the upper granular layers of the squamous epithelium. The virus is not believed to be cytolytic and virus is released in the upper layers of the epithelium. These terminally differentiated cells cannot proliferate and are shed from the epithelium within a day or so [Stoler *et al.*, 1992].

Viral replication is extra-chromosomal but in HPV-associated malignant transformation the viral DNA may be integrated [Cullen *et al.*, 1991], which often results in deletion of large sectors of the viral genome. The viral genome must linearise to splice into the host DNA. This frequently occurs within E1 and E2 viral genes, which are usually lost with L1 and L2 [Stoler *et al.*, 1992]. The E1 and E2 proteins are important because they regulate the transcription of other open reading frames (ORFs) in the early region of the genome. Loss of E2 function leads to the derepression of the viral promoter and a resultant enhanced expression of E6 and E7 oncoproteins, which are expressed in large amounts [Smotkin and Wettstein, 1987], incurring the production of E7-specific antibody [Park *et al.*, 1998]. In the circular replicating form of the viral genome, there is expression of only small quantities of E6 and E7. Over expression of the E6 and E7 proteins from oncogenic types, HPV-16 and –18, but not from HPV-6 or –11 [Gage *et al.*, 1990], overcomes the regulation by the cell of proliferation by the p53 protein and the retinoblastoma protein (Rb), by binding to these proteins [Imai *et al.*, 1991]. This allows uncontrolled cell growth and the potential for malignant progression [Munger *et al.*, 1989; Arends *et al.*, 1998].

The E6 protein acts by binding to p53 [Werness *et al.*, 1990] and directing its degradation and E7 binds and inactivates the Rb protein [Munger *et al.*, 1989]. Both Rb and p53 are cellular growth regulators, controlling the change from G_0/G_1 to S phase, with p53 also involved in responding to DNA damage by inducing growth arrest or apoptosis. Loss of their function promotes cell survival after DNA damage, allowing accumulation of genetic changes which could drive the progression to malignancy [White *et al.*, 1994]. It is apparent that neoplastic transformation is accompanied by mutations in 5 or more key mitotic regulatory proteins and this is accelerated by the removal of the function of p53 [Street and Delgado, 1995]. The expression of both E6 and E7 proteins is required to maintain the transformed state of the cell. The E5 protein is also an oncogene, but the E5 gene is often lost after integration, so its role in carcinogenesis is uncertain. There is evidence that the E5 protein may participate in the initiation of carcinogenesis [Howley, 1996] and to be involved in CIN lesions [Banks and Matlashewsli, 1996].

1.4. HPV infection and cervical disease

Genital HPV infections are transmitted mainly by sexual contact, possibly via small abrasions on the epithelial surface of the genital areas, this would allow HPV virions from the epithelial cells of one partner to invade the basal cells of the other. Most HPV infections disappear within months to a few years after diagnosis [Hildesheim *et al.*, 1994]. HPV infection of basal or parabasal cells may result in a latent infection [Schneider, 1990] where viral DNA resides in the nucleus and is replicated along with cell division. These cells appear morphologically normal and the latent infections are detectable only by the demonstration of HPV DNA. A latent infection may become productive but it is not known to what degree virus is shed, and the signal needed to induce replication is unknown [Ferenczy *et al.*, 1985]. Latent infection may be related to the immune state of some individuals. Infection in some individuals results in viral replication, which is achieved by subversion of the cell's own processes by the viral proteins. The virus is non-cytolytic and depends on the desquamation and cell death by apoptosis for release. There is no inflammation accompanying viral development, the innate immune system is not alerted and there is therefore a delay in the host's immune response. This is evidenced by the 8-12 months delay in the appearance of serum antibodies after HPV infection [Carter *et al.*, 1996]. The majority of cervical HPV infections clear spontaneously, possibly due to the

host's immune system interacting with the virus [Morrison *et al.*, 1991]. It is not understood why some individuals clear infection and others have persistent infection or why in some, HPV infection progresses to a neoplastic state.

If an infection is not cleared, infected individuals may have a persistent disease for a long time (several decades) or progress to high grade CIN and then invasive cancer, however this is uncommon. (Fig.1.4.). It appears that 5-19% of infected individuals who are not treated will develop CaCx in their lifetime [Richart *et al.*, 1998]. So most infections are asymptomatic, but lesions that do occur, appear 3 to 8 months after infection [Oriel, 1971]. A review of world literature [Östör, 1993] indicated that for CIN 1 the likelihood of regression is 60%, persistence 30%, progression to CIN3 10%, and progression to invasive CaCx 1%. For CIN 2 the likelihood respectively is 40%, 40%, 20%, and 5%. The likelihood of CIN 3 regressing is 33% and progressing 12%.

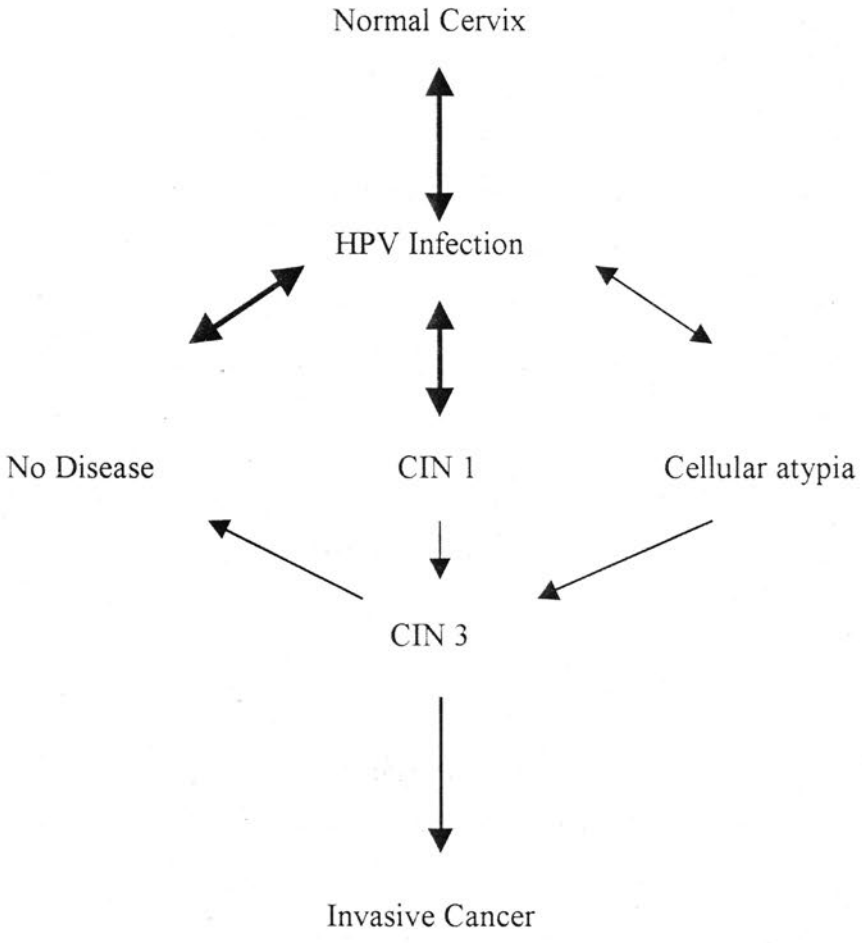

Fig. 1.4. A hypothetical description of the multistep pathogenesis of cervical cancer (redrawn from Schiffman and Brinton, 1995).

Most CINs and invasive carcinomas develop from the epithelium covering the cervical ectopy [Arends *et al.*, 1998]. This is exposed tissue that before puberty is found in the lower endocervical canal to the vagina. The columnar epithelium of this " transformation zone" undergoes metaplasia to a stratified squamous epithelium and it is at this time that the epithelium seems at most risk for developing intraepithelial neoplasia as a result of infection by viruses, or possibly other factors [Shah and Howley, 1996]. CIN is recognised by the presence of cytological atypia. In South Africa as in parts of Europe, CIN is histologically divided into three grades (Table 1.1) [Richart, 1967]

Table 1.1. The different terminologies used for squamous cell cervical lesions

Lesion	Classical Histology	LSIL/HSIL*
No CIN	Non-neoplastic normal-reactive	Negative
CIN 1	Neoplastic slight dysplasia	LSIL including koilocytosis
CIN 2	Moderate dysplasia	LSIL including koilocytosis
CIN 3	Severe dysplasia, carcinoma *in situ* (CIS)	HSIL
Invasive cancer	Invasive cancer	Invasive cancer

* Bethesda System, LSIL low-grade squamous intraepithelial lesion. HSIL high-grade squamous intraepithelial lesion. Adapted from Ponten *et al.* [1995].

In CIN 1 the lesion is a well-differentiated intraepithelial neoplasm and in CIN 3 the lesion is a poorly differentiated neoplasm, with CIN 2 in between (Fig 1.5.). There are cytopathic changes in the keratinocytes, recognised as the presence of HPV, such as koilocytosis and epithelial multinucleation [Koss and Durfee, 1956]. These changes are most conspicuous in CIN 1 and 2 and minimal in CIN 3 and might reflect viral integration in the high-grade lesions. Most invasive carcinomas will develop from the intraepithelial neoplasm that has formed in the cervical ectopy [Anderson *et al.*, 1991]. The discussion hereafter will be of squamous cell carcinomas and not adenocarcinomas, which constitute only a small proportion of cervical cancers [Pontén *et al.*, 1995]. Fig 1.5. represents the different terminologies used for CaCx precursors.

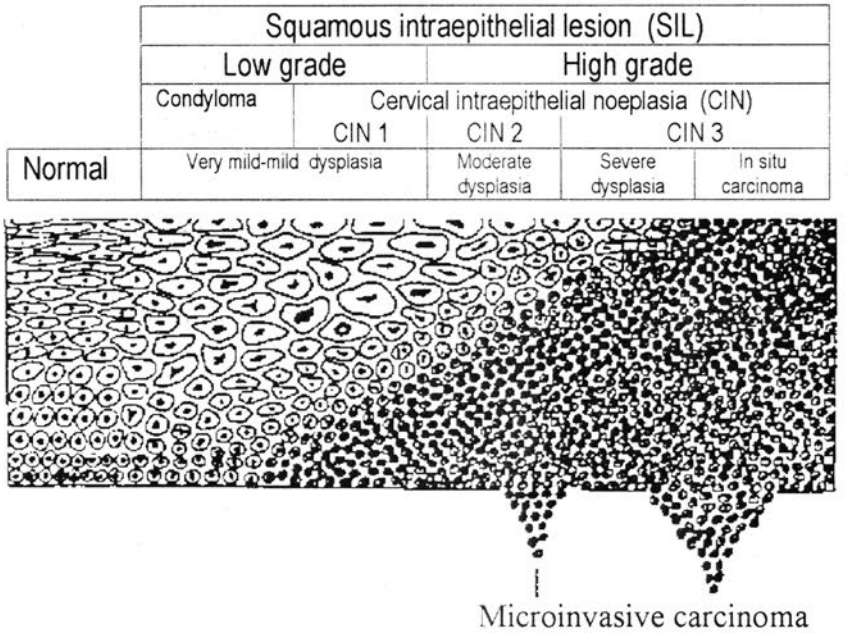

Fig. 1.5. A schematic representation of the cervical cancer precursors and the different terminologies used to refer to them. (Adapted from Shah and Howley [1996])

1.5. HPV types and their association with cervical disease.

The majority of HPV types infecting the cervix do have oncogenic potential but since the transformation to cancer is rare the frequently used term *high risk* type, for an oncogenic HPV type has been discouraged [Richart *et al.*, 1998]. HPV infects almost all the surface epithelia of the body, with the mucosal surfaces of the genital tract being infected by mucosotropic HPV types. HPVs infecting the female genital tract fall into two main types, those associated with benign lesions and those associated with cervical cancers [Lorincz *et al.*, 1987]. The types HPV-6b and HPV-11 or low-oncogenic-risk types (and indeed most genital HPV types) are associated with low-grade squamous lesions such as cervical condylomas and CIN 1, which is the morphological evidence of a productive HPV infection. The oncogenic types, mainly HPV-16, -18, -31 and 45 are found in 50-80% of CIN 2 and CIN 3 lesions and up to 90% of invasive CaCx [Lorincz *et al.*, 1992; Bosh *et al*, 1995]. There is a third group of less frequently detected types or intermediate-oncogenic-risk types (eg. HPV-33, -35 –39. –51, -52, -55, -58, -59, -68), which are found associated with all grades of CIN and occasionally with CaCx. More than 30 HPV types have been found in cervical samples [Richart *et al.*, 1998] (Table1.2.).

Table 1.2. Anogenital human papillomavirus types associated with disease*

HPV type	Disease association	Oncogenic association
6	Condylomata acuminata, low grade CIN, laryngeal papillomas	Low or no oncogenic risk
11	Condylomata acuminata, low grade CIN, laryngeal and conjunctival papillomas.	Low or no oncogenic risk
16	CIN1-3, Bowenoid papulosis, Bowen's disease, cervical, vulval, penile and anal cancers and precursors	Oncogenic risk
18	High grade cervical, vulval, penile and anal cancer precursors. Rarely low grade cancer precursors	Oncogenic risk
30	CIN	Low oncogenic risk
31	CIN1-3, cancers	Oncogenic risk
33	CIN1-3, cancers	Intermediate oncogenic risk
34	Bowen's disease; CIN	Intermediate ncogenic risk
35	CIN1-3, cancers	Intermediate oncogenic risk
39	Bowenoid papulosis;CIN ;cancer	Intermediate oncogenic risk
40	Bowenoid papulosis, Condylomata; CIN	Low oncogenic risk
42	Flat condylomata, Bowenoid papulosis; CIN	Low or no oncogenic risk
43	Condyloma; Low grade CIN	Low or no oncogenic risk
44	Condylomata acuminata; CIN	Low or no oncogenic risk
45	Condylomata; CIN; cancers	Oncogenic risk
51	Low grade CIN	Low or no oncogenic risk
52	CIN1-3; cancers	Intermediate oncogenic risk
53	Weak association with cancers	Low or no oncogenic risk
54	Condylomata acuminata	Low oncogenic risk
55	Penile condyloma; Bowenoid papulosis	Low oncogenic risk
56	Condyloma acuminata CIN1-3, cancers	Oncogenic risk
57	Low grade CIN	Intermediate oncogenic risk
58	High grade CIN; cancers	Oncogenic risk
59	Vulval IN	Moderate oncogenic risk
61	Vulval IN; CIN	Intermediate oncogenic risk
62	Vulval IN	Intermediate oncogenic risk
64	Vulval IN	Intermediate oncogenic risk
66	Cancers	Oncogenic risk
67	Vulval IN	Intermediate oncogenic risk
68	Genital lesion	Low oncogenic risk
69	CIN	Oncogenic risk
70	Vulvar papilloma	Low oncogenic risk

* [Richart *et al* 1998;Myers *et al.*, 1996; Howley *et al.*, 1996] Oncogenic risk = high risk

Epidemiological evidence indicates that up to 40% of young sexually active women are infected with HPV and can be infected with more than one type [Lunga *et al.*, 1992], although only a small percentage develop clinical lesions. Most HPV DNA-positive lesions regress spontaneously with a minimal host inflammatory response proir to the onset of regression [Coleman *et al.*, 1994], and only a small proportion progress to malignancy [Evander *et al.*, 1995]. The change from productive infection to carcinoma results in an increase in the mitotic rate of the cell and aneuploidy which is predictive of cancer [Winkler *et al.*, 1984]. Progression to neoplasia can take several decades [IARC/WHO, 1995] and is dependent upon the occurrence of several risk factors that impact on the outcome of the HPV lesion.

The virus type is the most important risk factor (Table 1.2.). Oncogenic types, HPV-16, HPV-18, HPV-45 and HPV-31 cause about 80% of CaCx in the developing world [Bosch *et al.*, 1995]. However, oncogenic HPV have been detected in a range of asymptomatic controls (3-30%), indicating development to neoplasia probably involves additional factors such as viral persistence or altered expression of viral genes.

1.6. Risk factors for HPV-associated neoplasms

There are a number of factors that enhance the chance of HPV infection resulting in malignancy. There are virus and host-related factors, as well as environmental influences that increase the risk of progression to cervical cancer.

1.6.1. Virus type

Infection with certain HPV types mainly HPV-16, as reviewed in the previous section, remains the most significant risk factor for the development of CaCx [Lorincz *et al.*, 1992; Richart *et al.*, 1998]. Occasionally viral genomes have been isolated which have mutations which result in the increased expression of their transforming genes [May *et al.*, 1994], increasing their oncogenic potential.

1.6.2. Host factors

An individual's genetic predisposition and the involvement of host genes appear to play a role in determining risk of CaCx. There is a significant familial clustering of cases of cervical cancer, which is more likely to result from a genetic rather than an environmental factor, but the causative chromosome locus has yet to be found [Magnusson *et al.*, 1999]. Host gene polymorphism has been implicated in the progression of HPV lesions by Storey *et al.* [1998], who claims that a p53 arginine allele instead of proline at the polymorphism at codon 72, provides a seven-fold higher risk of malignant conversion. This polymorphism has been found by others to not affect the chance of malignancy [Ngan *et al.*, 1999]. Chromosomal abnormalities increase with lesion severity, increasing the risk of neoplastic progression [Larson *et al.*, 1997; Greenspan *et al.*, 1997; Southern *et al.*, 1997; Mitra, 1999]. Much attention has been paid to the status regarding the human leukocyte antigen II (HLA II) of the infected individual. This part of the immune system presents antigens to effector T and B cells. There has been a demonstration of an increased CaCx progression in individuals with HLA II haplotypes of the DQ class [Odunsi *et al.*, 1996; Hildesheim *et al.*, 1998], an indication of the importance of the individuals immune system in the control of infection. HLA class 1 haplotype also appears important as an HLA-B7 genotype is associated with a poorer clinical outcome in CaCx patients [Ellis *et al.*, 1995; Hildesheim *et al.*, 1998]. These patients have a variation in the E6 protein sequence of the infecting HPV-16 that alters the HLA-B7 peptide binding epitope, which probably alters immune recognition by cytotoxic T lymphocytes (CTLs). This demonstrates how HPV-16 could escape immune recognition in HLA-B7 individuals. An HLA-B63 genotype has also been shown to increase an individuals susceptibility to CaCx [Krul *et al.*, 1999].

Immunodeficiency results in an increased susceptibility to HPV infection and an increased chance of progression to malignancy [Petry *el al* 1994]. Renal transplant patients on long term immunosuppressive drug regimes, have a greater incidence of HPV infection and genital neoplasia [Halpert *et al.*, 1986; Alloub *et al.*, 1989; Leigh *et al.*, 1999]. Women with human immunodeficiency virus (HIV) infection are at greater risk of HPV infection [Ho *et al.*, 1994; Arany and Tyring *et al.*, 1998] and CIN [Cappiello *et al.*, 1998; Goodman *et al.*, 1999]. The physiological immunosuppression of pregnancy leads to an increased risk of replicative HPV infections, with the virus becoming latent postpartum [Richart *et al.*, 1998]. There is evidence too of a decreased humoral immune response to HPV-16 antigens during pregnancy, however the significance of this with regard to malignant progression has yet to be determined [Sethi *et al.*, 1998].

1.6.3. The influence of a woman's number of sexual partners on CaCx

Genital HPV infection is a sexually transmitted disease, thus the amount of sexual activity with different partners would influence the exposure to and acquisition of HPV and therefore the progression to CaCx. Women commencing sexual relationships at an early age (>16 years), are at higher risk of CaCx than women starting later [Rotkin 1967; Herrero *et al.*, 1990]. A woman's number of sexual partners rather than the frequency of intercourse increase the risk of CaCx [Rotkin, 1967; Herrero et al., 1990; Schiffman and Brinton, 1995]. A man's sexual activity and the number of sexual partners he has (especially prostitutes) would increase the risk of cervical disease in his wife [Bosch et al., 1996]. Comparison of CaCx in Columbia and Spain, where there is an eight-fold higher incidence in Columbia than Spain, reveals this high incidence might be due to endemic HPV infection in that country [Bosch *et al.*, 1992; Nonnenmacher *et al.*, 1995]. This high HPV endemicity in Columbia is thought to be due to the high number of sexual partners reported in the male population compared to those reported for Spainish men. The latter is one possible explanation of the wide variation in the incidence of CaCx worldwide, which cannot be explained by the different possible co-factors in HPV infection required for progression to neoplasia.

1.6.4. Exogenous factors

Much attention has been focused on the detection of possible exogenous environmental influences contributing to neoplastic progression such as chemicals, UV light, smoking, oral contraceptive use, nutrition, and other viral or parasitic infections of the genital tract [Campo, 1998]. There is no confirmed evidence of the effects of diet, oral contraceptives, pregancy or other infections of the genital tract [Schiffman and Brinton, 1995]. There is a consistent epidemiological association between cigarette smoking and cervical cancer and a dose-dependent increased risk of CIN 2 or 3 in women with mildly abnormal cervical smears [Daly *et al.*, 1998]. A potent tobacco-specific carcinogen, nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has been found in the cervical mucus of cigarette smokers in significantly higher doses than in nonsmokers. This further strengthens the association between CaCx and tobacco smoking [Prokopczyk *et al.*, 1997].

1.7. Other HPV-associated mucosal cancers

1.7.1. Cancers at anogenital sites other than the cervix

HPV is not only associated with cancers of the cervix. There are many other genital sites that HPV will infect. The anorectal junction is quite similar to the transformation zone of the cervix [Fisher, 1994] and supports HPV-related lesions. Palefsky *et al.* [1991] established that 85% of anal cancers were HPV positive, with HPV-16 accounting for the majority of anal cancers [IARC/WHO, 1999]. Anal lesions infected with HPV-16/18 are associated with a neoplastic histology and HPV-6/11 with benign condyloma. There is an increase of anal intraepithelial neoplasia (AIN) amongst homosexual men and HPV detection is increased amongst these men who are HIV positive [Kiviat *et al.*, 1993; Hagensee *et al.*, 1997]. Male partners of women with CIN frequently have similar penile lesions and the same types as their partners and there is evidence of HPV in penile cancer mostly being associated with HPV-16/18 [Maden *et al.*, 1993]. Vulva intraepthelial neoplasias are also associated with HPV [Fisher, 1994], with vulval carcinomas occurring in younger women. Vulval carcinomas have been found associated with HPV-16, -18, and types -6, -11, and –30 associated with vulval condyloma acuminata [Traiman *et al.*, 1999].

1.7.2. Cancers of the head and neck region

HPV infection has been found associated with numerous tumours in regions other than the anogenital areas, especially recently with the improvement of HPV DNA detection techniques. In the eye, at least half of epithelial tumours of the ocular surface and lacrimal drainage system and certain inflammations of the eye have been shown to be HPV associated [Assadoullina *et al.*, 1999]. Syjranen *et al.* [1982] first demonstrated the presence of HPV in oesophageal cancers. This has subsequently been confirmed in high-risk areas such as China [Suzuk *et al.*, 1996; Lavergne and de Villiers, 1999] and South Africa where HPV DNA was found in 71% of oesophageal lesions [Williamson *et al,* 1991] and 52% of oesophageal lesions [Cooper et al., 1995]. But not in low-risk areas such as Japan [Akutsu *et al.*, 1995]. The association of HPV and oesophageal carcinomas has been controversial with Smits *et al.* [1996] finding no association of HPV with oesophageal carcinoma in the Netherlands, but Benamouzig *et al.* [1992] finding a 42% prevalence of

HPV in oesophageal cancers in France. A seroepidemiological study in Sweden, found no positive association between oesophageal squamous cell carcinomas or adenocarcinomas and antibodies to HPV-16 or HPV-18 [Lagergren *et al.*, 1999].

Reports regarding the presence of HPV in oral tumours have also been inconclusive until recently. Aggelopoulou *et al.* [1999] reported HPV DNA in 49% of oral tumours, with 22% containing HPV-16 and 44% HPV-18. Mineta *et al.* [1998] supported this in an extensive study investigating the association of HPV-16 and HPV-18 with head and neck squamous carcinomas. HPV-16 was found associated with 23% of all cases and HPV-18 with 4% of cases. These included carcinomas of larynx, nasal and paranasal sinuses, hypopharynx, oral cavity and oro- and naso-pharynx. HPV-16 and -18 were found most prevalent in carcinomas of the oropharynx (54%) and larynx (38%). A recent report has lent further support to the aetiologic role for HPV in head and neck squamous cell carcinomas [Gillison *et al.*, 1999]. Carcinomas of the larynx have been shown to be associated with HPV by Garcia-Milian *et al.* [1998] and Koufman and Burke [1997]. Nasal papillomas are rare but HPV-57b has been shown to be associated with these papillomas and carcinomas [Wu *et al.*, 1993]. A recent study of the HPV prevalence in squamous cell carcinomas of the upper aerodigestive tract, found a 30% prevalence of HPV in pharyngeal tumors, a 15% prevalence in laryngeal tumours and a 10.3% prevalence in buccal tumors [Pintos *et al.*, 1999]. HPV detection was however found not to be of prognostic value in these patients.

1.8. Infection in children by oncogenic HPV types

Laryngeal papillomas, associated with HPV-6 and 11, are found in children [Mounts and Shah, 1984] but information has been accumulating with regard to the presence of the oncogenic genital HPV types in children [Sedlecek *et al.*, 1989; Jenison *et al.*, 1990; Cason *et al.*, 1992; Cason *et al* 1995a]. Alternative modes of transmission of HPV other than sexual have been suggested [Cason *et al.*, 1995b], as HPV DNA has been detected at oral and/or genital sites of many children with no apparent clinical symptoms of disease [Mund *et al.*, 1998; Cason *et al.*, 1998; Rice *et al.*, 1999]. The nature of the subclinical infection however is as yet unknown. There is evidence of transmission from mother to child at birth [Sedlecek *et al.*, 1989; Pakarian *et al.*, 1994; Puranen *et al.*, 1997; Cason *et al.*, 1995a; Rice

et al., 1999] but evidence too, that horizontal transmission is feasible [Lacey, 1996]. Sedlacek *et al.* [1989] demonstrated the presence of HPV DNA in the oral cavity of 47.8% of newborn children delivered vaginally from mothers who had HPV DNA in their cervical cells and HPV DNA in their amniotic fluid. Pakarian *et al.* [1994] also demonstrated transmission of HPV-16 and HPV-18 from mother to child (55%), with 6 of 11 infants having persistent buccal HPV DNA at 6 weeks old. Cason *et al.* [1995a] were able to demonstrate a transmission rate of 73% (HPV-16 69%; HPV-18 76.9%) from mother to infant, a persistence of HPV-16 DNA in 79.5% at 6 weeks old and a persistence of HPV-16 DNA in 83.3% at 6 months old (HPV-18, 20%). The Cason *et al.* [1995a] study also detected anti-HPV-16 L1 and L2 IgM antibodies in children aged 5 to 16 years. Puranen *et al.* [1997] supported previous findings with a report of 37% of the nasopharyngeal aspirates of newborn infants having the same HPV DNA as their mothers. The topic is controversial however, with a recent review indicating that oncogenic HPV types are not spread vertically [Dillner *et al.*, 1999] and others [Cason et al., 1998; Rice *et al.*, 1999] suggesting that these HPV types are transmitted from mother to child.

Antibodies to oncogenic HPV types (mainly HPV-16) have also been described in children. Jenison *et al.* [1990] detected antibodies in children to recombinant proteins encoded by the E2, E7, L1 and L2 ORF of HPV-16 and –18 by Western Blot and found a 40% prevalence for any HPV-16 protein and a 28% prevalence for HPV-18. There have been more recent reports of antibodies to a peptide from the E2 protein of HPV-16 in children [Marais *et al.*, 1997; Hamsikova *et al.*, 1998] with a decreasing prevalence with age and there are other reports of early HPV-16 protein antibodies in children. Müller *et al.* [1995] reported a 20% seroprevalence of antibodies to an E4 peptide in children, which correlated significantly with the antibody prevalence to an E6 peptide. Hamsikova *et al.* [1998] reported a 30% seroprevalence of E4 antibodies in young children, and Mund *et al.* [1997] a 20.3% E4 antibody seroprevalence. Controversy does exist as to the presence of these antibodies as there are no clear clinical manifestations. The high levels of E2 and E4 antibodies in children have been declared likely cross reactivity with cross-reacting virion epitopes of other HPV types (e.g. HPV-1 and –2) [Mund *et al.*, 1997]. Cutaneous types, HPV-1 and HPV-2, are common in children but antibodies to HPV-1 have been found most prevalent in the age group 8-11 years [Anisimova *et al.*, 1994], whereas the HPV-16 antibodies most prevalent at age 3 years [Marais *et al.*, 1997; Hamsikove *et al.*, 1998]. Cross-reactivity with HPV-1 does therefore not appear likely. However, cross reactivity with other HPV

antigens could be possible although none have been confirmed. The presence of antibodies to virus-like particles (VLPs) have also been detected in children, to HPV-16 VLPs (VLP-16) [Mund *et al.*, 1997; Cubie *et al.*, 1998] and VLP-16, -18, and –33 [Hamsikova *et al.*, 1998; af Geijersstam *et al.*, 1999], albeit at low levels. Also sexually inexperienced girls, before the onset of sexual activity, have showed IgA and IgG seroresponses to VLPs of HPV types 6/11, 16 and 18 [Vandoornam *et al.*, 1998].

Genital warts, associated with HPV-6 and HPV-11, have also been described in children [Lacey, 1994; Bingham, 1994; Armstrong and Handley, 1997; Allen and Siegfried, 1998] with the incidence increasing in prepubertal children [Armstrong and Handley, 1997]. Genital warts in children have been linked with sexual abuse, although perinatal infection and indirect transmission by foamites, or hetero-inoculation have also been suggested. The question of the infection of genital HPV types in children does remain a topic requiring urgent clarification, especially with the proposed introduction of HPV vaccines. It will be important to assess to what extent antibodies in children will affect their resistance to HPV infection later in their adult life and the success of proposed vaccination strategies.

1.9. Distribution of HPV types worldwide

In a comprehensive study of the prevalence of HPV in cervical cancers worldwide, Bosch *et al.* [1995] were able to verify that HPV was associated with > 93% of cervical tumours. The study also established that there existed no significant variation in percentage prevalence among the 22 countries studied. This is unexpected when one notes the large variation in the incidence of CaCx worldwide. HPV-16 was the most prevalent type found worldwide, evident in 50% of specimens, with HPV-18 at 14 %, HPV-45 at 8% and HPV-31 at 5% (Fig. 1.5a). HPV-16 was the most prevalent type in all countries except for Indonesia where HPV-18 was most common (48.9%). There was an increase in prevalence of HPV-45 in the West African countries, Guinea (33.3%) and Mali (17.2%) and in East Africa, Tanzania (10.2%). HPV-39 and HPV-59 were confined almost entirely to Central and South America. There is a reported high prevalence in Tanzania [Bosch *et al* 1995; ter Meulen *et al.*, 1992] and Uganda [Schmauz *et al.*, 1989] of HPV-18, (17.5% and 35%, respectively) in CaCx cases, compared with other parts Africa and of the world (Fig. 1.5b). and can be compared with the prevalence in South Africa (Fig. 1.5c.).

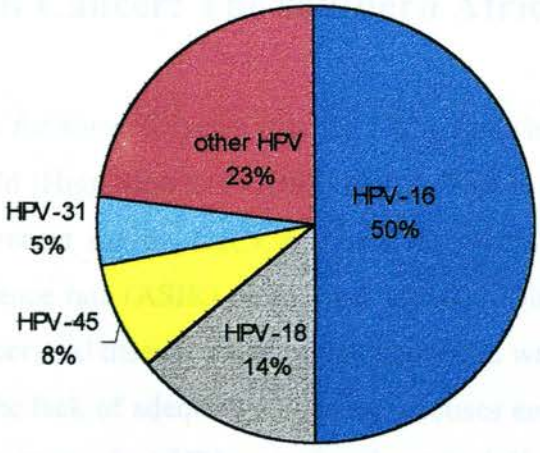

Fig 1.6a The prevalence of HPV types in CaCx patients world wide [Bosch *et al.*, 1995]

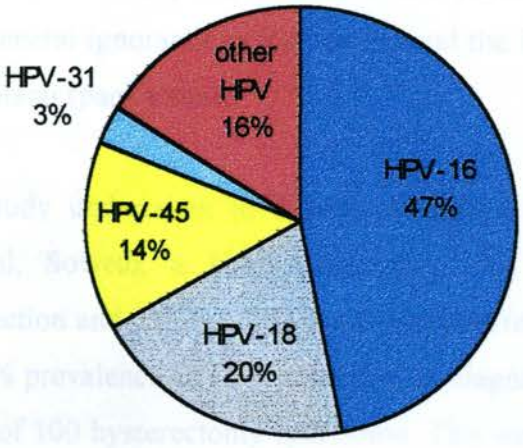

Fig. 1.6b. The prevalence of HPV in CaCx patients in parts of Africa (Algeria, Benin, Guinea, Mali, Uganda and Tanzania [Bosch *et al.*, 1995].

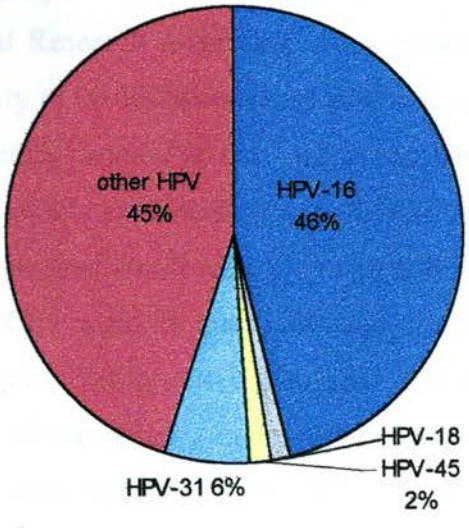

Fig 1.6c. The prevalence of HPV in women with CaCx in South Africa [Williamson *et al.*, 1994].

1.10. Cervical Cancer: The southern African perspective

It has been known for some 40 years that the CaCx rates in South Africa are amongst the highest in the world [Higginson and Oettlé, 1960]. This is especially so for black women, where in a more recent survey, CaCx accounts for 31.2% of all cancers and has an age standardised incidence rate (ASIR) of 26.5 per 100,000 [Sitas *et al.*, 1996]. The extremely high incidence of cervical disease in the region compared with the rest of the world [Pontén *et al.*, 1995] and the lack of adequate screening practises emphasised the pressing need for a prophylactic vaccine against HPV associated cervical disease in southern Africa. Most black women present at local hospitals with advanced disease, due to socioeconomic and cultural factors and general ignorance of the disease and the fact that they are not routinely screened by Papanicolaou (pap) smears.

In an histological study undertaken in 2 separate patient groups (1972 and 1982) at Baragwanath hospital, Soweto, a 100% association was found between histological evidence of HPV infection and CIN3 in 200 conization specimens [Markowitz *et al.*, 1986]. There was also a 66% prevalence of HPV infection as diagnosed by histology in a control group, without CIN, of 100 hysterectomy specimens. This was well above the then highest reported HPV prevalence rate in the world of 36.5%. Of all human cancers, only that of the cervix has the potential for successful control by screening for cervical abnormalities [Prorok *et al.*, 1984]. A program was undertaken by the Cytology Unit of the South African Institute of Medical Research to provide mass screening for women in Soweto, aiming at a screening capacity of 90.000 women per annum [Leiman, 1987]. A preliminary study was instigated to identify women at risk and factors for the delay in diagnosis of cervical disease, which was mainly evidenced as poor patient knowledge. In the pilot study of 1000 women, those women aged 40-60 were pinpointed as targets for screening. In this group, detection rates for CIN were 28 per 1000 and 13 per 1000 for CaCx, figures unequalled in world literature. The project failed after 3 years but extrapolation of the results obtained to the rest of the country, indicated the project could have enabled the detection of at least 400 invasive and 900 in-situ CaCx cases per annum. A major reason for failure was the low priority afforded to patient screening by the administration of health services.

Bloch *et al.* [1988] undertook a study to assess the prevalence of HPV and herpes simplex virus (HSV) in 2 isolated communities in South West Africa (SWA), now Namibia. The population groups were mainly Herero, but also Himba and San (Bushmen) and the opportunity for sexual contact with outsiders for these people was considered remote. Here, as in South Africa, the prevalence of HPV infection and CIN was found amongst the highest reported in the world. HPV infection by cytology was 24% in the women examined, 83% of these showed pre-malignant or more advanced lesions. The highest incidence of HPV and CIN was in the 20-39 year old group, 85.6% and 91.7% respectively. Factors favoring the development of CIN and CaCx were the early commencement of sexual activity, multiplicity of sexual partners and the presence of HPV and HSV.

Studies in this department examined the prevalence of HPV DNA in biopsy material from CIN 3 in patients from Cape Town by Southern blot hybridisation and HPV typing with specific probes [Williamson *et al.*, 1989]. A large diversity of HPV types were detected in women with CIN 3 (HPV types 6, 11, 18, 31, 33 and 35) with a lower incidence of HPV-16 (16%), than had been found in similar studies in Europe and the United States. Johnson *et al.* [1991] studying condyloma acuminata biopsies from women in South Africa, using Southern blot and type-specific probes, found the prevalence of HPV-6a, HPV-11a and two new subtypes of HPV-6 and -11 in these biopsies. Up until then there had been no data on the prevalence of the HPV types associated with cervical disease in South Africa.

Studies in Durban using *in situ* hybridisation, compared Durban patients with CIN and CaCx to those in Britain [Cooper *et al.*, 1991a; Cooper *et al.*, 1991b] and concluded that HPV types vary geographically with more of the "minor" types (other than type 16), being more common in South Africa. It was found that HPV-16 was the predominant type in women with CIN2/3 with a 44% prevalence in South African women and a 68% prevalence in the British women [Cooper *et al.*, 1991a.]. In the South African specimens, 36% of those that were HPV positive were HPV-33 or HPV-35, and 19.4% were HPV-18. There was no HPV-31, -6 or –11 found in the CIN biopsies [Cooper *et al.*, 1991a.]. Using *in situ* hybridisation and biopsy material from CaCx patients, Cooper *et al.* [1991b] found a 42% prevalence of HPV-16 and a 22% prevalence of HPV-18. No HPV-6, -11, -31, -33 and –35 were found in any of the cancers.

Further work in Cape Town to determine the incidence of HPV types in CaCx samples using polymerase chain reaction (PCR) and Southern blotting [Williamson *et al.*, 1994], reported the first incidence of HPV types 45, 52 and 58 in Africa. HPV type 16 (HPV-16) was found in 46% of CaCx patients, HPV-18 in 1.5% and HPV-31 and –33 in 6% of patients each. Ramesar *et al.* [1996] studied women with normal cervical cytology in Cape Town for the presence of HPV DNA using Southern blot hybridisation. A 13% HPV DNA and a 9% prevalence of oncogenic HPV types were found with HPV-18 being the most prevalent type, constituting 28% of the total HPV types detected.

1.11. Role of the immune system in HPV infection

The key role of the immune system in the control of HPV infection and progression to cancer is becoming more evident. The immunocompetance of an individual has an impact on their ability to deal with HPV infection and on the progression of HPV-associated lesions. Renal transplant patients will have a 17-fold greater incidence of genital HPV infection compared with normal controls [Alloub *et al.*, 1989]. HIV infection provides an important example of the effect of immunosuppression on HPV infection. HIV infection is associated with a very high prevalence of HPV DNA detection [Ho *et al.*, 1994], a higher rate of persistent HPV infection and an increase in cervical disease [Sun *et al.*, 1997], compared with HIV-negative individuals. Several clinical and experimental studies have shown that cell mediated immune responses play a role in both susceptibility to and regression of HPV infections [Coleman *et al.*, 1994; Frazer *et al.*, 1986]. However, HPV-associated lesions can persist for years in immunocompetent individuals. There is a long delay in HPV infection before the appearance of HPV-specific antibodies, compared with the appearance of antibodies to lytic viruses [Cubie, 1972]. A delay of 6-12 months after infection has been noted before antibody to the viral capsid protein is detectable [Carter *et al.*, 1996], so HPV is not detected by the immune system and eliminated in the conventional way as with viruses causing cell lysis. There is evidence that HPV in its co-evolution with its host, has produced ways to evade the immune system by avoiding certain specific and non-specific immune responses [Frazer *et al.*, 1999]. The site of infection of HPV in the skin, renders it less available to immune regulation. A proposed mechanism HPV uses to evade the immune response, is the delay of the expression of the highly immonogenic L1 and L2 proteins to the superficial layers of the epithelium distant from

immune cells and which are shed after a few days [Stoler *et al.*, 1992]. This is achieved by the use of rare condons by the capsid protein genes as discussed in section 1.3., that results in inefficient translation in the undifferentiated basal epithelial cells, where there are not sufficient of the required tRNAs available [Zhou *et al.*, 1999].

1.11.1 Humoral immunity to HPV

In the past, data related to the humoral immune response to HPV has been inconsistent and difficult to interpret due to the variability of the various assays and the choice of study populations. Serological assays for HPV have been based on fusion proteins, peptides, virions and VLPs [Bonnez *et al.*, 1991; Cason *et al.*, 1992; Dillner *et al.*, 1994; Galloway, 1994; Nonnenmacher *et al.*, 1996]. These studies have attempted to link the seroresponses to specific HPV antigens with the cervical disease status of women [Dillner, 1994; Schiller and Roden, 1995; Lehtinen *et al.*, 1996; de Gruijl *et al.*, 1997] and to identify markers of cervical disease. HPV serology displays inherent problems as there are a number of HPV types, the majority of which are not sexually transmitted or associated with malignancy. Different epitopes may cross react between types. The rate of induction of antibodies during the course of HPV-induced disease will probably be different, with some responses developing fairly rapidly after infection and others only later when tumours develop [Wikström *et al.*, 1995; Carter *et al.*, 1996; Muller *et al.*, 1992]. Antibodies will fluctuate due to regression of lesions or the reactivation of latent infection [Wikstrom *et al.*, 1995]. Depending on the epitope used in a study, serological results will therefore be different with regards to type-specificity and disease association. In an assay using a defined epitope (as in a specific peptide) it is possible to separate epitopes which are not type-specific for cervical disease from those that are type-specific. The use of whole proteins or VLPs, which contain a number of epitopes will only provide information on disease association if there are immunodominant epitopes that are disease and type specific.

There has been substantial progress recently in the understanding of humoral immunity against HPV as a result of the availability of HPV proteins, peptides and VLP for immunological studies. Many large studies of HPV serology have demonstrated that antibodies to HPV-derived epitopes are associated with CIN or CaCx [Hamsikova *et al.*, 1994; Dillner *et al.*, 1994; Muller *et al.*, 1995; Dillner *et al.*, 1995]. The antibody response

against HPV antigens may be variable for different HPV types, for different epitopes and different host responses [Park *et al.*, 1998]. These factors should be borne in mind when assessing serological results. Epitope mapping studies, using overlapping synthetic peptides or sets of truncated fusion proteins produced detailed maps of linear epitopes of various ORFs of HPV-16 [Cason *et al.*, 1989; Dillner *et al.*, 1990; Dillner, 1990; Muller *et al.*, 1990; Krchňák *et al.*, 1990]. Epitopes that were preferentially reactive to antibodies from patients with cervical disease were described. Epitopes in the E2 protein [Dillner, 1990; Dillner *et al.*, 1989; Lehtinen *et al.*, 1992; Dillner *et al.*, 1994], E4 and E6 protein [Dillner, 1990; Muller *et al.*, 1992; Le Cann *et al.*, 1995], and E7 protein [Dillner *et al.*, 1990; Muller *et al.*, 1992; Jochmus-Kudielka et al 1989] as well as parts of the L1 or L2 ORFs [Cason *et al.*, 1992; Müller *et al.*, 1990; Dillner *et al.*, 1994; Le Cann *et al.*, 1995] were used in Western blot or enzyme-linked immunosorbant assays (ELISA). These assays found antibodies to a number of these HPV-derived proteins to be more common in sera of patients with cervical disease than healthy controls. The results however, differed markedly using the similar or the same proteins and peptides.

1.11.1.1. Antibodies to the E2 protein

The E2 gene encodes proteins involved in the regulation of viral transcription and is often disrupted during the linearisation of the viral genome prior to its integration into the host genome [Baker *et al.*, 1987]. Peptides to seroreactive regions of the E2 protein of HPV-16 and HPV-18 have been used extensively in ELISA to identify E2-specific antibodies in women with cervical disease. Differences in relative risks (RR) were found for CIN and CaCx compared with controls, using the same E2 peptide of HPV-16 (designated 245-16, aa 328-345) in ELISA or an equivalent E2 peptide of HPV-18 (245-18). These are presented in Table 1.3. The strongest association was found for IgG to the equivalent peptide based on the E2 of HPV-18, E2-245-18, with a RR of 9.2, in women with CaCx. The reasons for the incompatible results have been given as differences in the assays used and also the different methods of selection of cases and controls [Dillner, 1994].

Table 1.3. Summary of some major studies using the E2 peptides, 245-16 or 245-18, to detect antibodies in women with cervical disease.

Epitope and Ig	Disease studied	Relative risk	P value	Reference
245-16 IgA	CIN	3.4	0.002	Dillner *et al.*, [1989]
245-16 IgG	CIN	2.1	0.005	Dillner *et al.*, [1989]
245-16 IgA	CIN	6.0	0.007	Dillner *et al.*, [1994]
245-16 IgA	CaCx	9.5	0.03	Lehtinen *et al.*, [1992]
245-16 IgG	CaCx	2.8	0.000	Dillner *et al.*, [1994]
245-16 IgG	CaCx	1.9	0.03	Mann *et al.*, [1990]
245-16 IgG	CaCx	2.4	0.004	Hamsikova *et al.*, [1994]
245-18 IgG	CaCx	9.1	0.00	Dillner *et al.*, [1994]
245-18 IgA	CaCx	2.3	0.001	Dillner *et al.*, [1994]

1.11.1.2. Antibodies to the E4 protein

The E4 gene, although located in the early region of the PV genome, is expressed as a late gene and the E4 protein has a role in productive infection and may aid virus release from the cell by collapsing the keratin cytoskeleton [Roberts *et al.*, 1993]. Antibodies to the E4 protein of HPV-16 have been found significantly more frequently in women with CaCx than normal healthy controls but differences in E4 antibody prevalence rates have been found. Initially, using fusion proteins and in Western blot assays, no difference was found in the prevalence of E4 antibodies between CaCx patients and controls [Jochmus-Kudielka *et al.*, 1989], although a high (33%) seroprevalence was found in children. Köchel *et al.* [1991] found a marked increase in anti-E4 antibodies of HPV-16, in CaCx patients compared with normal healthy controls. Muller *et al.* [1995], using an E4 peptide (amino acids,aa 33-47) in ELISA, found a low prevalence of anti-E4 in adults (16.2%) but a high seroprevalence in children (30.4%), supported by a significant association with anti-E6 positivity and concluded that E4 antibodies may be a marker of virus replication. Vonka *et al.* [1999] have shown a significant association of anti-E4 (and E2) antibodies with CaCx, where CaCx patients possessed antibodies more frequently than controls. The relevance of the evidence of the association between CaCx and antibodies to various HPV proteins and E2 and E4 peptides in all these studies was difficult to assess. The reason being, not all

infected individuals had detectable antibodies, and there was no correlation between serological data and the HPV DNA status of the individual.

1.11.1.3. Antibodies to the E6 and E7 proteins

The prevalence of antibodies to the oncoproteins HPV-16 E6 and E7 (E7-16) in sera of patients with cervical cancer have produced more notable results. These proteins are consistently expressed in cervical cancer cells [Baker *et al.*, 1987], which is accompanied by inflammation and cell death below the epithelial basal membrane and the production of E7 and E6-specific antibodies. The presence of antibodies to both these proteins have been found to be markers of invasive cancer. The strongest association was found for the E7 protein in radioimmunoprecipitation assay (RIPA) and a synthetic E7 peptide (aa6-35) in ELISA [Sun *et al.*, 1994; Baay *et al.*, 1997]. Antibodies to E6 and E7 *in vitro* translated proteins are barely detectable in serum of patients with CIN [Park *et al.*, 1998]. Mann *et al.* [1990] detected E7-16 antibodies in 25% of CaCx patients and 6% of controls in ELISA with a synthetic peptide corresponding to the whole protein. Krchňák *et al.* [1990] described similar findings with a synthetic peptide derived from the N-terminal portion of E7 (aa11-30). Using fusion proteins and Western blotting, Jochmus-Kudielka *et al.* [1989] and Köchel *et al.* [1991] found a 14-fold and 6-fold higher prevalence of E7-16 antibodies respectively, in patients with CaCx than in healthy controls. There was a strong concordance between results obtained using E7 protein in Western blot or synthetic E7 peptide (aa11-30) in ELISA in parallel [Suchánková *et al.*, 1991]. CaCx patients reacted to a broader range of 3 overlapping HPV-16 E7 peptides than controls [Hamšivová *et al.*, 1994] and in the case of the E7-16 derived peptides an association was shown with HPV-16 DNA [Hamšivová *et al.*, 1994]. The most widely used antigen in E7 assays has been the HPV-16 E7 (aa6-35) peptide or the whole E7 protein, with seroreactivity to the E7 protein being described as a more discriminating marker of CaCx than peptides [Muller *et al.*, 1992]. However, the presence of antibodies to E7 peptide (aa 6-35) in the sera of patients with CaCx correlates with size of the lesion, lymph node involvement and a poor prognosis [Baay *et al.*, 1995]. This peptide is of prognostic value in early-stage CaCx, conferring a 6 to 7-fold increase in the risk of death [Viladiu *et al.*, 1997]. Vonka *et al*, [1999], studying the antibody prevalence to HPV-16 early antigens, E2, E4 and E7 in women with CaCx, and those that developed the disease during the course of the study and matched controls,

found seroprevalence to peptides derived from all the early proteins in CaCx patients. Development of disease was associated with seroconversion to an HPV antigen in the majority of women, but not significantly. This was in line with other observations that antibodies to HPV early antigens (E2, E4, E6, and E7) are only markers of high-grade disease [Dillner *et al.*, 1994; Hamsikova *et al.*, 1994; Baay *et al.*, 1997; Park *et al.*, 1998]. In summary, these serological markers were disappointing however, as even in those with HPV-16 positive cancers, only about 50% were positive for E7. The strongest association with E7 was found with late-stage disease [Baay *et al.*, 1995] and no association with carcinoma in situ, the precursor of CaCx.

1.11.1.4. Antibodies to L1 and L2 proteins

Discordant results have been described using peptides to the two capsid proteins, L1 and L2. In the L1 protein there is a well characterised epitope in its mid-region (L1:13), which has shown an association with CaCx [Dillner *et al.*, 1994; Dillner *et al.*, 1990]. Dillner *et al.* [1991], Cason *et al.* [1992] and Le Cann *et al.* [1995] reported conflicting results with similar L1 peptides of HPV-16 (aa 473-492). Cason *et al.* [1992] found antibodies to this peptide in 91% of CIN patients who were HPV-16 DNA positive, Le Cann *et al.* [1995] found this peptide only weakly reactive with sera of women HPV-16 positive and Dillner *et al.* [1990] also found this peptide only weakly antigenic. In the L2 protein, a highly reactive epitope has been described, (L2:49), which was not found to be associated with CaCx [Dillner *et al.*, 1994] but IgA antibodies to this epitope were found associated with condylomas [Wikström *et al.*, 1992]. Further capsid peptides were found to be non-reactive or only weakly associated with cervical disease [Dillner, 1994]. These data however, all suggest that viral proteins are immunogenic and may represent targets for the immune surveillance of HPV-related cancers. It is the antibodies to conformational epitopes which appear to be the main component of the human immune response to HPV infection [Bonnez *et al.*, 1991; Christensen *et al.*, 1992]. A study of antibodies to conformational epitopes on VLPs has had the widest application in the study of the immune response to HPV infection and its association with CaCx.

1.11.2. Virus-like particles and the immune response

The PV capsid is composed of two structural proteins L1, the highly conserved, major structural protein and L2 as discussed in section 1.2. L1 is a 55-kD protein, which is stable in two oligometric configurations, capsomeres that are pentamers of L1 and capsids composed of 72 capsomeres. So the virion contains 360 L1 molecules arranged as 72 capsomeres on a T=7 icosahedral lattice (Fig.1.1.), each made up of 5 L1 molecules [Baker *et al.*, 1991]. The L2 protein or minor protein of 74-kD, occurs in quantities 5-10 times less than L1 [Orth *et al.*, 1978]. The L1 protein contains species-specific conformational (located externally) and genus-specific (located internally) epitopes. Epitopes on L2 have also been identified, indicating that at least some of the L2 molecule is exposed on the virion [Heino *et al.*, 1995]. Condylomas and low grade CIN lesions usually express L1 [Bistoletti *et al.*, 1988] but L1 is infrequently expressed in high grade CIN and rare in invasive cancers [Pfister, 1987]. It is possible that L1 induces humoral and cell mediated immune (CMI) responses to HPV infection [Rudolf *et al.*, 1999]. However, the limitation of the expression of L1 and L2 to the most superficial layers of the epithelium most likely reduces their potential for presentation to the immune system. This could be a strategy that PVs may have evolved to evade immune recognition (section 1.11.) [Frazer *et al.*, 1999].

Oncogenic type HPV virions are sparse in lesions, so are not available as an antigen source for serological detection of HPV. Experimental HPV-11 virions have been used [Bonnez *et al.*, 1991] to detect antibodies in women with genital warts, but the production of virions is laborious and limited in quantity. The expression of the L1 capsid protein in eukaryotic cells has resulted in the self-assembly of the protein into VLPs [Zhou *et al.*, 1991; Rose *et al.*, 1993; Hagensee *et al.*, 1993; Kirnbauer *et al.*, 1992]. These VLPs are indistinguishable from native virions under the electron microscope [Hagensee *et al.*, 1994] (Fig.1.7. colour plate page 139). Expression of both L1 and L2 in cells results in an increased yield of VLPs [Kirnbauer *et al.*, 1993; Hagensee *et al.*, 1993], which are more stable than L1 VLPs and are antigenically similar. VLPs composed of L1 are able to induce neutralising antibody levels similar to those induced by virions [Rose *et al.*, 1994a]. The L1 capsomeres can also induce high titer neutralising antibodies [Rose *et al.*, 1998]. There is little cross-reactivity between HPV types with immune sera to whole VLPs (species-specific epitopes) [Rose *et al.*, 1994b], whereas the genus-specific antibodies in sera made

with disrupted VLPs are cross-reactive [Christensen *et al.*, 1992; Heino *et al.*, 1995]. Volpers *et al.* [1995] identified epitopes on the L2 protein in HPV-33 VLP, indicating L2 could be involved in immune stimulation. These observations suggested that VLPs could be reliable substitutes for virions in immunological studies evaluating the immune response to conformational surface epitopes.

ELISAs developed using VLPs have demonstrated their utility as assays for markers of infection with HPV. Kirnbauer *et al.* [1994] established that 59% of women with HPV-16 infection had IgG antibodies to HPV-16 VLP (VLP-16) whereas only 6% of women negative for genital HPV DNA had detectable antibody levels. The assay was put forward as potentially useful in the determination of the natural history of HPV infection and the identification of women at risk of developing CaCx. However in this and subsequent studies some women with HPV DNA were judged seronegative in the corresponding type VLP ELISA. It was thought that some women did not mount a detectable serological response to infection or that some women tested may not have had time to mount a sufficient response. In support of this, it was observed that persistent HPV-16 infection, as measured by repeatedly being HPV-16 DNA positive, increased the likelihood of seropositivity in an individual [Wideroff *et al.*, 1995]. Wideroff *et al.* [1995] and Wikström *et al.* [1995] demonstrated that those women who no longer had detectable HPV DNA, maintained their HPV VLP seropositivity, implying that VLP seropositivity was a measure of prior exposure to HPV infection. Wikström *et al.* [1995] were able to show the induction of antibodies to several HPV antigens, including E2 245-16 and VLP-16 at the time of a new infection. Antibody titers were shown to decline after treatment or with regression of HPV-associated lesions [Bonnez *et al.*, 1993; Dillner, 1994]. It has been demonstrated that seropositivity to VLP-16 correlated with the lifetime number of sexual partners of HPV-16 DNA negative individuals [Wideroff *et al.*, 1996]. Antibodies to VLP-16, VLP-18 and VLP-33 were shown to correlate with sexual behavior [Dillner *et al.*, 1996], providing confirmation that the mode of transmission of HPV was sexual. This indicated that that HPV serology could be a marker of sexual behavior. VLP ELISAs appeared type specific [Heim *et al.*, 1995], although reactivity to VLP-16 was associated with cervical DNA of another type, although less strongly than with HPV-16 DNA in the absence of other types [Wideroff *et al.*, 1996].

The VLP-16-based ELISAs (and to a lesser extent with oncogenic HPV types, 18, 33) have been employed extensively to assess the risk association of HPV infection and CaCx. In a nested case-control study in women with CaCx and matched controls, the presence of IgG antibodies in ELISA against VLP-16 was found to have a predictive value for developing CaCx with an odds ratio (OR) of 12.5 [Lehtinen *et al.*, 1996]. In a large case-control study in Columbia and Spain, seropositivity was shown to be strongly associated with cervical disease with ORs of 3.8 and 56.7 respectively [Nonnenmacher *et al.*, 1995]. The difference in the OR reflected differences in the seroprevalence of the control groups. Columbia has an 8-fold higher incidence of CaCx, with a higher seropositivity amongst normal Columbian women, who also had a 3-fold higher HPV DNA prevalence than normal Spanish women. Approximately 25% of women with CIN 3 and genital HPV-16 DNA tested negative in this study reflecting the moderate sensitivity of the assay or lack of detectable antibodies. Further geographic variations in the prevalence of genital HPV infection compared with the incidence of CaCx have been described, leading to speculation as to the presence of other etiological co-factors in the determination of CaCx risk. Nonnenmacher *et al.* [1996] studied the differences in genital HPV-16 DNA and VLP-16 ELISA seropositivity amongst women attending sexually transmitted disease (STD) clinics in Greenland and Denmark. Greenland has a 5-fold higher incidence of CaCx compared with Denmark. It was shown that women at low risk of cumulative exposure to HPV were more likely to be HPV DNA positive than seropositive, and those at high risk cumulative exposure were more likely to be seropositive than HPV DNA positive. Greenlandic women who were HPV-16 DNA positive were mostly seronegative, whereas in Denmark there was no difference between the seropositivity of the HPV DNA positive and negative groups. They concluded that genital DNA decreased with cumulative HPV exposure in high-risk groups and that HPV DNA is not a marker for comparing exposure to HPV in both high- and low-risk populations. VLP ELISA seropositivity however, reflected relative cumulative exposure.

An important study by Carter *et al.* [1996] investigating the relationship between seropositivity to VLP-16 and HPV-16 infection in women attending a university, was able to show a median time for seroconversion after detection of HPV DNA as 8.3 months. The reason for the delay in seroconversion was thought to be due to the capsid protein avoiding immune recognition because of the inaccessible site of L1 protein expression in the upper layers of the squamous epithelium. The antibody titres were low but the average ELISA

optical density (OD) values were stable for the 3-year duration of the study. Women who seroconverted were more likely to develop HPV-16 associated CIN (RR=5.7), but most of the CIN cases detected were not related to HPV-16, indicating the need for a multi-HPV type serodiagnostic assay. To estimate the risk of developing CIN among women exposed to HPV, Chua *et al.* [1996] extended previous studies by assessing the seroresponses in 74 women from a population-based cohort study, who developed CIN to VLP-16, -18 and –33 compared with 148 who remained CIN free. VLP-16 seropositive women had a 3-fold increased risk of developing CIN, but no increased risk of CIN was found for seropositivity to VLP-18 or VLP-33. Wang *et al.* [1997a], assessing the seroresponses of CaCx patients and age-matched blood donors in VLP ELISA of HPV types 16, 18 and 33, found that IgG seropositivity to all three was associated with CaCx, including IgA seropositivity to VLP-16, although less strongly than IgG VLP-16 seropositivity. This study confirmed the >50% sensitivity of the capsid serology found in previous studies and that serology was not exclusively HPV-type specific (ie only approximately 50%) when relating the seroprevalence of antibodies of a specific HPV type to the HPV DNA status in the same individual.

The relationship between the virion antibody response and the course of cervical disease development with time had not been described until de Gruijl *et al.* [1997] examined the presence of anti-VLP-16 IgG in women with mild to moderate cervical dyskaryosis. These women were followed for up to 34 months. Women with persistent HPV-16 infections were found more likely to be VLP-16 seropositive and more likely to develop CIN 3. This indicated an increased risk of disease in those with anti-VLP-16 IgG antibodies and that these antibodies were not responsible for the clearance of CIN lesions, and that there must be other mechanisms involved in clearing cervical lesions. The HPV-specific response at the genital mucosa was suggested as the area to be examined for elucidation of the development of cervical disease. Most recent advances in VLP serology have indicated that it is a systemic IgA anti-VLP-16 response that correlates with the clearance of HPV-16 [Bontkes *et al.*, 1999] and that this response is a by-product of a successful cell mediated immune (CMI) response induced at local (cervical) lymph nodes. Local IgG and IgA, as determined in cervical secretions, did not correlate with virus clearance or with disease course. An extension of the same [Bontkes *et al.*, 1999] study showed IgG responses to be associated with HPV-16 persistence and high-grade CIN lesions [de Gruijl *et al.*, 1999] and also determined the relationship between T-helper (Th) cell–dependent Interleukin-2 (Il-2)

and the IgG response. The Il-2 response was shown in the majority of patients with CIN, and was independent of the HPV status and disease outcome, indicating that it was not responsible for the control of infection.

1.11.3. The role of cell mediated immunity

Humoral immunity may be responsible for the prevention of viral infection and inactivation of virus particles, but the destruction of HPV-harbouring cells and the regression of HPV-associated lesions appears to be cell mediated. The role of the cell mediated immunity (CMI) in HPV infection has been inferred from observations in individuals with compromised T cell function. People infected with human immunodeficiency virus (HIV) type 1 (HIV-1) and immunosuppressed allograph recipients show a greater prevalence of HPV infection and cervical disease [Sun *et al.*, 1997; Alloub *et al.*, 1989]. The classes of cells responsible for CMI are thought to be lymphoid cells which infiltrate skin or mucosal surfaces, local antigen presenting cells (Langerhans cells) and the epithelial cells themselves [Malejczyk *et al.*, 1997]. There is also a network of locally produced cytokines that play an important role in the cell-mediated control of HPV infection, down regulating HPV-expression and affecting the growth of HPV-infected cells. Impairment of cytokine function might result in increased tumour growth, as has been indicated in experimental systems [Malejczyk *et al.*, 1997]. Other deficiencies in host immune responses correspond with the progression of HPV-related disease. There are the reduced numbers of cervical Langerhans cells seen in some patients with cervical disease [Hawthorne *et al.*, 1988] and a down regulation of major histocompatibility complex (MHC) class 1 antigens in CaCx patients [Conner and Stern, 1990]. There is evidence too of MHC phenotypes which render the individual more susceptible to HPV-associated cancer (section 1.6.2).

It has been well documented that HPV infections are common in normal populations [Pfister *et al.*, 1987; Evander *et al.*, 1995; Grandilone *et al.*, 1996; Hildesheim *et al.*, 1994]], which is a characteristic of a latent infection, with the majority of infections being eventually cleared. Regression appears to represent the induction of an effective host response and these mechanisms involved in the regression of HPV-associated cervical lesions are being identified. Effective T cell responses have been shown in normal populations where peripheral blood mononuclear cells were found to proliferate in response

to HPV peptides corresponding to sequences from the L1 or E6 proteins of HPV-16 [Strang *et al.*, 1990] and also E7 proteins [Altmann *et al.*, 1992]. Scott *et al.*, [1999] studying cytokine patterns in HPV-positive women who cleared infection, demonstrated that expression of interferon gamma (IFN-γ) and interleukin–4 (IL-4) was associated with the clearance of infection, indicating that a T-helper 1 (Th1) type cell response was associated with clearance.

Comparing the proliferative responses to a number of peptides derived from the HPV-16 L1 in normal healthy individuals with responses in women with cervical dysplasia, Shepherd *et al.* [1996] identified immunogenic areas of HPV-16 L1 (aa 199-409) to which both groups responded with predominately $CD4^+$ T cell responses. CIN 3 patients were found more likely to respond to the immunogenic area, aa 311-345, than control groups and all patients with HPV-16 DNA positive biopsies responded to one or more of the peptides under study. Luxton *et al.* [1996] showed the levels of proliferative responses to both HPV-16 E7 and L1 to be reduced in women with CaCx compared with those with cervical dysplasia and healthy controls. These differences in T cell responses could reflect the difference in ability to control HPV infection and related cervical disease. Luxton *et al.*, [1997] further demonstrated the importance of the T helper (Th) response in the control of HPV infections. A Th1 type response was evident in healthy controls but reduced in women with cervical disease and a decrease in the Th1 type response corresponded with an increase in the severity of cervical lesion. Kadish *et al.* [1997] demonstrated that the proliferative responses of women with CIN to certain HPV-16 E6 and E7 peptides was associated with clearance of HPV infection and lesion regression.

Conversely, in a longitudinal study, de Gruijl *et al.* [1996] linked proliferative T cell responses to a fusion HPV-16 E7 protein and E7 peptides with viral persistence and progression to disease. That study was able to demonstrate that the percentage of women with proliferative responses to E7 was significantly higher in CIN patients with persistent infection which indicated that these responses do not reflect successful antiviral immune responses but that other factors were involved. More recently, de Gruijl *et al.* [1999] in a prospective study to determine the relationship between cellular (Th cell dependent IL-2 production) and the humoral (serum IgG) immune responses in women with CIN against VLP-16, found Th responses in patients with both viral clearance and viral persistence.

The serum IgG response correlated with an IL-2 response in 87% of CIN patients. That study also demonstrated that IgG responses were associated with HPV-16 persistence (section 1.11.2.), so neither cell-mediated or IgG responses to VLP-16 alone were sufficient to control HPV-16 infection and CIN development.

The studies on cytotoxic T lymphocyte (CTL) responses in cervical disease have concentrated on responses to the E6 and E7 proteins, which are the most highly expressed proteins in CaCx. Evans *et al.* [1997] demonstrated the presence of HPV-16 E7-specific CTLs in the peripheral blood and draining lymph nodes of CaCx patients and identified virus-specific CTLs infiltrating the HPV-induced tumor. Nimako *et al.* [1997] studied the HPV-16 and HPV-18-specific E6/E7 CTL responses in CIN 3 patients and found CTLs in 6/10 CIN 3 patients but none in normal healthy women. A recent study has shown that VLP-16 can stimulate a MHC 1 restricted CTL response with human peripheral blood lymphocytes *in vitro,* which was specific for VLP-16-infected target cells [Rudolf et al., 1999]. This finding could be important for the monitoring of the immune response to VLP based vaccines. However, the association with HPV-specific CTL and lesion regression has not been clearly demonstrated [Shepherd and Luxton, 1999] and thus the CTL response could be important in restraining cervical disease progression but this has to be proven.

The clearance of HPV-associated lesions has been shown to be characterised by an increase in macrophages and CTLs with the CD4-$^{+}$ lymphocytes predominating [Coleman *et al.*, 1994] and changes associated with regression were consistent with a delayed–type hypersensitivity (DTH) reaction to a foreign antigen. The events involved in the persistence of HPV-associated lesions have, however, not been confirmed. There is evidence that HPV-16 E7 induces T-cell tolerance in mice [Doan *et al.*, 1998; Borchers *et al.*, 1999] and humans [Doan *et al.*, 1999; Sheperd and Luxton 1999] which could cause suppression of the HPV-specific responses in CaCx patients and result in lesion persistence. However more research is required to clarify the cell-mediated events associated with lesion regression and persistence as well as the association of systemic immune events in these processes. The study of humoral immunity to HPV infection at the cervical mucosa and a closer assessment of events at the cervical mucosa, could assist in the elucidation of the mechanisms related to lesion persistence or regression.

1.11.4. Cervical Mucosal Immunity

There are two functionally different compartments of the immune system, the systemic system (bone marrow, spleen and lymph nodes) and the mucosal system (mucosal lymphoid tissue and external secretory glands) [Mestecky and McGhee, 1987]. A response in the one system does not necessarily mean a corresponding response in the other [Mestecky, 1987]. The genital tract mucosa is the first line of defence against invading HPV as it is their portal of entry and thus the induction of a cervical immune response may be important for immune protection against HPV. The endocervical mucosa has been demonstrated as a functional immunological system. The endocervix has Langerhans cells which possess a dendritic morphology, for antigen presentation and the most Ig-containing and Ig-secreting cells of the female genital tract [Crowley-Nowick *et al.*, 1995]. The cervical epithelial cells may also be involved in the presentation of antigen [Roche and Crum, 1991]. The major classes of lymphocytes are well represented, $CD4^+$, $CD8^+$ and B cells or Ig bearing cells. On immune antigenic challenge, a humoral immune response is induced which results in the activation of B cells (or plasma cells). The B cells release mainly dimers and polymers of IgA (pIgA) and these are then transported into the lumen of the mucosa where they operate as secretory antibodies [Brandtzaeg and Farstad, 1999]. The antibodies in secretions represent the first barrier to the entry of pathogens to the body.

1.11.4.1. The immunoglobulins of the genital mucosa

The profile of Ig isotypes in the genital mucosa was thought to be 30% IgG and 60% IgA [Ogra *et al.*, 1981], but more recent reports indicate that IgG is predominant over IgA, both being derived systemically as well as locally [Bouvet *et al.*, 1994; Mestecky and Fultz, 1999]. The superior protective ability of IgA over IgG has been demonstrated, provided IgA is in a polymeric form [Renegar *et al.*, 1998]. Secretory IgA polymers (S-IgA), are synthesised locally by subepithelial plasma cells, in lamina propria regions of the mucosa, as polymeric J chain-containing molecules (pIgA). They are then actively transported through epithelial cells after binding to the trans-membrane secretory component (SC) poly Ig receptor [Mestecky and McGhee, 1987] and they are released into the lumen as S-IgA bound to the SC. The role of SIgA seems to be of adherence to pathogens and their conveyance by the mucus stream and also the prevention of attachment of pathogens to the

cervical mucosa [Bouvet *et al.*, 1994]. This polymeric form of IgA (pIgA) is also 7-10 times more effective than monomeric IgA (mIgA, the most common form in serum) at virus neutralisation and also more effective than IgG [Renegar et al., 1998]. Polymeric IgA may also be found in serum but its origins have been uncertain. In animal experiments chronic exposure to antigens at mucosal surfaces results in the migration of IgA memory cells to bone marrow, where they give rise to IgA forming cells on antigenic challenge [Alley *et al.* 1986] which produce serum pIgA. This mucosa-bone marrow axis could exist in humans. Serum IgA and SIgA systems are reported as having a high degree of independence [Mestecky and Fultz, 1999].

IgA occurs in two subclasses, IgA1 and IgA2, of which there are equal proportions in the female genital tract, and which have different functions and vary in their distribution and protein and carbohydrate structures [Russell *et al.*, 1999]. Immunisation with protein (or viral) antigens tends to elicit IgA1 and immunisation with polysaccharides IgA2 antibodies [Russell *et al.*, 1999]. It also appears that not only the type of antigen but the duration of the response regulates the expression of the IgA subclasses as well as the synthesis of pIgA or mIgA respectively [Russell et al., 1992]. It is therefore not only at mucosal surfaces that pIgA is produced.

The IgG in vaginal secretions may also originate both from local plasma cells and from serum [Mestecky and Fultz, 1999]. It has been shown that parenteral vaccination with tetanus toxoid increases the IgG in vaginal secretions not explained by passive transudation from serum [Bouvet *et al.*, 1994], and both a systemic (bone marrow, lymph node) and local origin was indicated. Studies have shown that the concentrations of Ig in cervico-vaginal secretions are not constant. The proportion of IgG to IgA remains relatively constant, except during mid-menstrual cycle, when both increase 3 days before ovulation and decrease to lowest levels at the time of ovulation. The increase in IgA and IgG levels both correlate with the production of the cyclical reproductive hormones, oestradiol and progesterone, as well as local cytokines, IL-1β, IL-6 and IL-10 [Kutteh *et al.*, 1998], indicating a regulatory role of these hormones and cytokines.

1.11.4.2. Common mucosal immune system

The primary mucosal immune response is believed to initiate in structures of the mucosal-associated lymphoid tissue (MALT), which samples antigen from the mucosal surface [Brandtzaeg and Farstad, 1999]. The most studied system is the gut-associated lymphoid tissue (GALT), and locally primed B cells will migrate (or traffic) from GALT to other secretory tissues where they reside as IgA plasma cells or memory B cells [Mestecky, 1987]. There is evidence therefore in animals and humans for an integrated common mucosal immune system (CMIS) [Mestecky, 1987]. Mucosal immunisation (or antigenic stimulation) at one site is followed by the appearance of antibody-secreting cells in the blood and antibody responses at distant sites from the original site of stimulation. The cervical mucosa is thought to be part of this common mucosal response.

The CMIS can be divided into two functional compartments, inductive and effector sites, with the inductive sites in certain loci such as GALT, being represented by Peyer's patches. The origins of plasma cells that reside in the cervical glands have been traced in animals to organised lymphoepithelial structures (Peyer's patches) of the GALT and also of the peritoneal cavity [Scicchitano *et al.*, 1988] as well as nasal cavity, rectum and possibly the genital tract [McGhee *et al.*, 1999]. These cells mature in mesenteric lymph nodes and enter the circulation through the thoracic duct and lodge in the lamina propria of the genital tract (and intestine and respiratory areas) and in mammary, salivary and lacrimal glands. Here they differentiate into IgA plasma cells at these effector sites under the influence of locally produced interleukins (IL) (IL-5, IL-6 and IL-10) [McGhee *et al.*, 1989]. The lack of the identification of an inductive site in the genital tract suggests that the inductive sites present in other areas (nasal cavity, rectum, GALT), supply the mucosal effector sites of the genital tract with the precursor cells that produce antibodies or are involved in cell-mediated immunity [Hook *et al.*, 1999]. However, the vaginal immunisation route has been shown effective in inducing a local immune response in the female genital tract [Kozlowski et al., 1997] but not in a murine system [Haneberg *et al.*, 1994]. These contradictory observations imply important differences between species in the induction of antibody-producing cells. The lack of a vaginal IgA antibody response after vaginal immunisation in the mouse was explained as possibly being due to the lack of organised mucosal lymphoid tissue and M cells in the female genital tract. A vaginal IgA response in

the human after vaginal immunisation does suggest local inductive sites, but these have not been located.

1.11.4.3. Local cervical antibody response to HPV infection

Studies characterising the HPV antigen-specific, local antibody response in cervical mucosa and its relation to cervical disease have indicated that it does exist and that this response may correlate with the presence of disease [Dillner *et* al 1989; Wang *et al.*, 1996; Bontkes *et al.*, 1999]. It does however remain to be determined whether a local antibody response in humans is protective and whether it will prevent reinfection as has been described in animal model systems [Breitburd *et al.*, 1995; Balmelli *et al.*, 1998]. The level of local human antibodies detected has been very low, but the minimum required neutralising level is unknown. Recently Bontkes *et al.* [1999] investigated the relationship between local and systemic IgG and IgA responses against HPV-16 VLP and viral clearance. The study was able to show that systemic IgA responses were not accompanied by local IgA responses and that the systemic response correlated with the clearance of HPV-16 and the resolution of cervical lesions. The indications were too that HPV infection had to be sustained for at least six months for the VLP-specific IgA responses to be elicited. More work is necessary in determining the relationship between the systemic circulation and the mucosal site of cervical lesions to determine whether peripheral findings do reflect those of the lesions and the role of systemic immunity in the regression of lesions. It has been shown that local VLP-specific IgA declines rapidly after successful treatment whereas systemic IgA was more stable [Elfgren *et al.*, 1996]. Important to consider too is that mucosal antibody responses are normally short-lived (a few months to a year), whereas antibodies in serum can persisit for decades [Ahmed and Gray, 1996]. The reason of these differences in antibody responses in unknown but the short-lived mucosal response could have significant consequences for protective immunity against mucosal infections.

1.11.4.4. HPV vaccines and cervical mucosal immunity

The understanding of the stimulation of the mucosal immunity, especially at the cervix, is a critical area of research for the development of HPV vaccines. Most important to note is

that, the induction of a peripheral immune response by antigen which is systemically administered, does not always result in significant mucosal immunity [Belyakov *et al.*, 1999; McGhee *et al.*, 1999]. Mucosal immunity is preferentially induced by mucosal immunisation, and this fact highlights the division of the systemic and mucosal immune systems [Belyakov *et al.*, 1999]. There is also evidence of subcompartmentalisation within the common mucosal immune system [Kantele *et al.*, 1998]. Cell distribution throughout this common mucosal system may not be uniform and certain inductive sites may give rise to responses preferentially manifested at certain effector sites or there may be relocation back to the original site of stimulation. The fact that there may be compartmentalisation within the CMIS [Moldoveanu et al., 1995] i.e. that immunisation at certain inductive sites may give rise to a humoral response which is preferentially manifest at certain effector sites only, is important to consider for vaccine studies [McGhee *et al.*, 1999]. Animal and human studies have indicated various sites of immunisation for maximal genital antibody stimulation. There is controversy with regards to the route which best evokes secretions of IgA in the female genital tract [Bergmeier *et al.*, 1995; Kantele *et al.*, 1998; Rudin *et al.*, 1998; Balmelli *et al.*, 1998]. Vaginal plus oral immunisation with Simian immunodeficiency virus (SIV) gag p27 linked to the yeast retrotransposon VLP, effectively induced SIV-specfic IgA and IgG in vaginal fluid and serum and IgA in saliva [Bergmeier *et al.*, 1995]. Nasal immunisation in mice (with VLP-16) and humans with cholera toxin B subunit (CTB) was found superior to the oral route in inducing genital antibodies [Balmelli *et al.*, 1998; Rudin *et al.*, 1998]. Oral immunisation of women with *Salmonella typhi* Ty21a was favoured over rectal immunisation for eliciting specific antibodies in vaginal secretions [Kantele *et al.*, 1998]. More recent evidence using oral, rectal and vaginal immunisation with CTB in women indicated that each immunisation route similarly increase anti-CTB IgG antibodies in serum and IgA in saliva. Only vaginal immunisation increased specific antibodies in genital tract secretions and likewise rectal immunisation only increased rectal antibodies [Kozlowski *et al.*, 1999]. The optimal routes of immunisation of the female genital tract are being further investigated [Kutteh, 1999].

1.12. Virus-like particles and vaccine prospects

There has been considerable interest in developing vaccines to prevent infection with HPV. VLPs are the immunogen chosen by most for vaccine studies [Kirnbauer, 1996a]. They do not contain potentially dangerous DNA and are highly immunogenic, eliciting high titres of neutralising antibodies [Kirnbauer *et al.*, 1992; Rose *et al.*, 1994a]. A major immunodominant antigenc determinant has been characterised in the majority of human sera positive for HPV-16 DNA, reactive to VLP-16 [Wang *et al.*, 1997b]. This activity was blocked with the type-specific monoclonal antibody (Mab), V5. The occurrence in some sera of non-neutralising antibodies, not blocked by V5, could be of pathologic significance in disease development but this has to be determined. It was suggested that the exposure of this epitope (and a similar one, R5 for HPV-18) would be a desirable property of a vaccine against HPV-16 or HPV-18.

The papillomavirus diseases of animals have proved valuable models for the development of vaccine strategies in humans. Papillomaviruses are species–specific and using the appropriate VLP, protection against PV-induced disease has been demonstrated. Suzich *et al.*, [1995] reported complete protection against canine oral papillomavirus (COPV) induced tumours after vaccination with COPV VLP. Similar protection has been described for rabbits and cows vaccinated against cottontail rabbit papillomavirus (CRPV) and bovine papillomavirus type 4 (BPV4) respectively [Breitburd *et al.*, 1995; Kirnbauer *et al.*, 1996]. It has been shown that neutralising antibodies are largely responsible for the protection, as passive transfer of serum from immunised animals conferred protection to the recipient against viral challenge [Breitburd *et al.*, 1995; Suzich *et al.*, 1995]. The demonstration of neutralising antibodies in sera and cervical secretions of African green monkeys after systemic immunisation with VLP-11 [Lowe, *et al.*, 1997] was important as it could mean that VLP vaccination would work in humans. Indications are that a multivalent vaccine will be necessary to cover the major genital HPV types causing CaCx as antibodies to VLPs appear type specific. White *et al.* [1998] have shown a limited cross protection between HPV types in the *in vitro* neutralisation analyses using polyclonal sera. Chimeric VLPs vacccines are an exciting prospect for therapeutic vaccines. Using chimeric VLPs, an early HPV protein may be fused to L1 (or L2) and animal models have shown the regression of established tumors in vaccinated animals or protection against challenge from

a tumor cells line expressing HPV early proteins [Greenstone *et al.*, 1998]. A single dose of VLPs resulted in successful vaccination without adjuvant. It has been recently demonstrated that VLP-16 could induce an HPV-16-L1-specific T cell response in human peripheral blood lymphocytes, that was MHC class 1 restricted [Rudolf *et al.*, 1999], and which would allow the monitoring of the immune response to VLP vaccines in humans. The most important consideration at present is to determine a vaccine protocol that will induce the maximal mucosal immunity and whether it is neutralising antibodies at this site or local CTL, or both which will be responsible for protection.

A recent report [Gellin, 1998] outlined the potential HPV vaccines, produced by pharmaceutical and biotechnology firms, at present being tested in clinical trials. MedImmune has developed a prophylactic vaccine for genital warts, MEDI-501. This consists of HPV-11 VLP with an alum adjuvant which is similar to the successful COPV vaccine used to immunise beagles [Suzich *et al.*, 1995]. Phase I trials were began in 1997 with MEDI-501, using healthy volunteers and a placebo-controlled, dose escalating trial. The pharmaceutical company, Merck has also prepared a VLP vaccine for clinical trials, but this is multivalent, containing VLP types -6, -11, -16 and –18.

The recent report of the International Agency for Research on Cancer/WHO (IARC/WHO) technical meeting on the development of HPV vaccines [IARC/WHO, 1999], was able to present results from some of the VLP vaccine clinical trials. A VLP-11 vaccine in alum (Medimmune) was well tolerated, induced high titres of antibody with cross-reactivity in a VLP-6 ELISA and HPV-11 neutralising titres of 1000 or higher in 33/34 individuals. A VLP-6 vaccine, given without adjuvant to individuals with genital warts, also induced antibodies and DTH responses. A VLP-16 vaccine in the early stage of clinical trials, was shown to induce significant antibodies in normal volunteers. The technical meeting emphasised the importance of inducing specific mucosal as well as systemic immunity for L1 vaccines to be effective, and that efforts are being made to study mucosal immunity in human vaccine trials. It was hoped that prophylactic vaccine efficacy trials would be initiated within a few years. These would require large numbers of individuals studied over a long period, and would probably start with young women commencing sexual activity. A successful vaccine would have to protect against a number of different HPV types to relieve the massive disease burden.

1.13. The aims and objectives of this study

There is a paucity of information in South Africa with regard to infection by genital HPVs and how this relates to cervical disease. Information available with regard to the most prevalent genital types in the southern African region, and those associated with cervical disease is limited and nothing is known about the immune response in South African women to genital HPV types. This study aimed to detect the degree of exposure to HPV in South African women and to establish which HPV types were associated with cervical disease. By so doing it, was hoped a better understanding of the immune response to HPV infection would be attained. The data was important too for the development of HPV vaccines and the establishment of vaccination strategies. The detection of individuals infected in the past would be a significant factor in the interpretation of results of future immunisation studies in an area of high HPV endemicity as South Africa. To do this, assays were developed to test the nature of the immune response in serum to various peptides derived from HPV antigens and in serum, cervical secretions and oral fluid using HPV VLPs to ascertain which antigens most successfully detected HPV exposure and predicted cervical disease.

Serum antibodies to several HPV antigens are generally induced at the time of an HPV infection [Wikström *et al.*, 1995] and a number of serological assays have detected antibodies to various HPV antigens to be more prevalent in women with cervical disease than normal healthy women. To determine whether this was so in South African women, antibodies in sera of normal women were compared with antibodies in sera of hospital patients with CIN and CaCx. The prevalence of antibodies to the 5 HPV VLP types causing the most CaCx worldwide [Bosch *et al.*, 1995] was assessed in patients and prevalence rates compared with normal blood donor controls. A unique opportunity to examine the nature of the serological response to 5 HPV types in a relatively isolated group of people, the San, enabled the assessment of HPV exposure in a defined community. Children were also examined to determine their serum antibody responses to HPV antigens, as little was known about antibodies to oncogenic HPV types in children.

Apart from the serological determination of antibody responses to prevalent HPV types in the region, this study aimed to further examine the humoral response to HPV infection.

HPV infects the cervical mucosal surface and a study of the local immune response at the cervix could more precisely reflect the cervical HPV infection. The examination of the anti-VLP IgA and IgG antibody responses in cervical secretion of women with cervical disease aimed to determine whether these assays were superior to serological assays in determining markers of cervical disease. The study of cervical HPV antibodies aimed to resolve significant factors about the local immune response to HPV infection. As a mucosal antigenic stimulus is reflected at other mucosal areas as part of the common mucosal immune system [Mestecky, 1987], it should also be possible to detect HPV antibodies at other mucosal surfaces. The oral mucosa is part of the common mucosal immune system. The study of the presence of oral antibodies to HPV proteins aimed to further examine the nature of the mucosal antibody response to HPV infection and to develop a non-invasive technique for HPV antibody detection. An oral test for antibodies to HPV has not been previously reported. To extend the study, antibodies were assayed for in serum, oral fluid and CM of the same CIN patient and compared with their HPV DNA status both at the cervix and in the mouth. A study of a number of samples from the same women with cervical disease aimed at obtaining significant insights into the immune response to HPV in these women.

Women testing HIV seropositive (HIV-positive), have an increased prevalence of HPV infection [Sun *et al.*, 1997] and the immunosuppressed state of these women is believed to be the reason for their inability to clear HPV infection. There have been no reported studies of the antibody responses to HPV infection in HIV-positive women. A study conducted on the HPV antibody prevalence in a group of commercial sex workers, 50% of whome were HIV-positive, hoped to determine differences in the immune responses of the HIV-positive women which contributed to their increased HPV infection. The sex workers were at high risk of HPV infection, with between 7 and 40 partners per week. A comparison of the antibody responses in serum and cervico-vaginal lavage (CVL) samples between the HIV-positive and –negative women hoped to detail differences in their immune response to HPV infection. The nature of the humoral response to HPV in immunodeficient compared with immunocompetent women might assist in the definition of a normal immune response to HPV infection.

Cell mediated immunity is also considered important in the control of HPV infection and the induction of a cell mediated immune response as well as a humoral response is

considered beneficial for a vaccine against HPV. HPV is host specific and will not infect laboratory animals. It was necessary to develop an animal challenge model for testing the nature of the cellular immune response to virus-like particles. This study also aimed to use the mouse challenge model to assess the efficacy of potential HPV vaccines to protect against viral challenge.

CHAPTER 2. ANTIBODIES TO HUMAN PAPILLOMAVIRUS TYPE 16 E2 AND L1 PEPTIDES AND HPV-16 VIRUS-LIKE PARTICLES AS SEROLOGICAL MARKERS OF DISEASE

Women with CIN have an age-related seropositivity to an E2-16 peptide and HPV-16 virus-like particles.

2.1. INTRODUCTION

Serological tests to detect HPV-specific antibodies should provide insights into the natural history of HPV infection and provide serological markers of the progression of HPV associated diseases. The HPV E2 protein has roles in virus replication and transcription, and loss of E2 function may be related to the progression of cervical neoplasia [zur Hausen, 1994]. It is therefore of interest to monitor immune responses to the E2 protein and its involvement in cervical disease. Epitope mapping studies as described in Chapter 1.11.1., have identified epitopes on the E2 protein which preferentially react with antibodies from patients with cervical disease [Dillner, 1990; Dillner *et al.*, 1989; Lehtinen *et al.*, 1992; Dillner *et al.*, 1994]. One E2 peptide in particular has been used extensively to identify antibodies in women with CIN and CaCx (Chapter 1.11.1.1.). This is an E2-derived synthetic 19-residue peptide from the carboxy terminal of the E2 protein of HPV-16 (defined as 245:16), and has been used in ELISA to determine seroconversions [Wikström *et al.*, 1995] and the association of HPV-16 antibodies with CIN [Dillner *et al.*, 1989]. Antibodies to this E2 peptide of HPV-16 and to its homologous peptide from HPV-18 E2, sharing identity in 10 of the 19 positions, (termed 245:18), have also been shown to be associated with CaCx [Dillner *et al.*, 1994]. Some characterised epitopes in the L1 region [Dillner *et al.*, 1994; Dillner, 1994] induce antibodies that are preferentially seen in CaCx and CIN patients [Cason *et al.*, 1992; Dillner *et al.*, 1994]. A peptide based on a poorly conserved area situated near the carboxy terminus of the L1 protein of HPV-16 (aa 437-492), has been described by Cason *et al.* [1989] (Chapter 1.11.1.4). Hydrophilicity chart analysis indicated a major hydrophilic peak in this region, suggesting a dominant B-cell epitope. Cason *et al.* [1992] described a 91% prevalence rate of antibodies to this peptide in women with CIN who were HPV-16 DNA positive.

Recent studies have demonstrated that the presence of antibodies to conformational epitopes on specific HPV VLPs can be used as a marker of cervical infection [Rose *et al.*, 1994a; Kirnbauer *et al.*, 1994] (Chapter 1.11.2.). Antibodies to VLP-16 have also been described as predictors of disease [Dillner *et al.*, 1995; Nonnenmacher *et al.*, 1995; Carter *et al.*, 1996; Lehitinen *et al.*, 1996]. Serological VLP tests appear to be type-specific, as individuals with DNA of a certain HPV type are more likely to have antibodies to that specific type [Kirnbauer *et al.*, 1994; Hamšiková *et al.*, 1997; Wideroff *et al.*, 1995]. Since 50-85% of patients with the detectable DNA of a certain HPV type will develop antibodies to the same HPV type, the ELISA tests have been validated. VLP-16 and VLP-18 exhibit a major type-specific conformational epitope, which can be neutralised [White *et al.*, 1999]. Type specific monoclonal antibodies (H16.V5 and H18.V5) were shown to block the serological reactivity of the majority of human sera to the corresponding VLP-16 or VLP-18 capsids [Wang *et al.*, 1997b]. This implied that the use of intact VLPs in ELISA should detect a type specific response, which is unlikely to be cross-reactive.

In the light of the paucity of serological data pertaining to HPV infection in Southern Africa, the aim of this study was to determine the prevalence of antibodies to HPV antigens and how they related to cervical disease. Three groups of people (children, blood donors and women with CIN) were tested for serum IgG levels to three antigens from HPV-16, one antigen from HPV-18 and one from bovine papillomavirus type 1(BPV-1) as a control. The HPV antigens were chosen because of their proven preferential reactivity with antibodies from women with cervical disease. The antigens comprised an HPV-16 L1 peptide [Cason *et al.*, 1992] and two E2 peptides, 245:16 and 245:18 [Dillner *et al.*, 1994] and BPV-1 VLP (BPV-VLP) [Ghim, 1996] and HPV type 16 VLP (VLP-16). All these antigens were used in ELISA to provide information on the prevalence of HPV antibodies in the South African populations and to determine serological markers for CIN.

2.2. MATERIALS AND METHODS

2.2.1. Serum samples

Sequential serum samples were drawn from 95 patients with CIN attending a colposcopy clinic at Groote Schuur Hospital, Cape Town, South Africa, during 1994 by gynaecologists, L. Denny, R. Soeters, C. Dehaeck and J. Nevin. Cervical punch biopsies were taken at the same time as the serum samples. Histological assessment was performed by the Department of Anatomical Pathology at the University of Cape Town. Disease was graded in 74 patients as CIN 3, in fifteen as CIN 2 and in six as CIN 1 by standard histological criteria [Richart, 1968]. Age-matched controls within the age range of five years, were selected from female blood donors at the Western Province Blood Transfusion Service. The average age of the women was 37 years. One hundred and fifty-five children's sera were obtained from patients at Red Cross Children's Hospital, Rondebosch, Cape Town and from private pathologists in Cape Town. These sera had been submitted to the laboratories for routine diagnostic tests unrelated to HPV. Ages ranged from 2-12 years. There were seventy-six female children and seventy-one male children. The gender of eight of the children was not recorded.

2.2.2. HPV Antigens

2.2.2.1. Peptides:

The three peptides used in this study were selected by virtue of positive reactivity with sera from women with cervical disease.

E2-245-16 (19 mer) (HKSAIVTLTYDSEWQRDQC) [Dillner *et al.*, 1989],

E2-245-18 (19 mer) (EKTGILTVTYHSETQRTKC) [Dillner *et al.*, 1994],

L1-16 (19 mer) (LKAKPKFTLGKRKATPTTS) [Cason *et al.*, 1992]

These were designated E2-16, E2-18, and L1-16 respectively. For single and three letter amino acid designations see appendix 1. The peptides were synthesised by Genosys USA.

2.2.2.2. Virus-like particles

The VLP-16 and BPV-VLP preparations were provided by Dr. Robert Rose, Rochester University, Rochester, USA. For the production of VLP-16, HPV-$16_{Rochester}$, L1 was cloned using methods previously described [Rose *et al.*, 1993], from viral DNA recovered from experimentally induced HPV-16 lesions propagated in the severe combined immunodeficiency (SCID) mouse [Bonnez *et al.*, 1998]. VLP were expressed from recombinant baculoviruses as described by Rose *et al.* [1990 and 1993].

2.2.3. Enzyme linked immunoabsorbent assays (ELISA)

Peptide ELISAs were performed as described by Dillner *et al.* [1990] with some modifications. Preliminary tests were performed to establish the optimal concentrations of the various reagents and of the sera to be tested. Various blocking agents were tried, alone and in combination. These included bovine serum albumin (BSA), fetal calf serum, blocking agent (Boerhinger Mannheim) and milk powder (Elite). The most effective blocking agent was found to be a 1% milk powder in PBS (Appendix 2) containing 0.05% Tween-20 (M/PBS-T) and was used in all subsequent ELISA tests. Microtiter plates (Nunc-immuno, MaxiSorp C, Roskilde, Denmark) were coated with peptide at a coating concentration of 20μg/ml as described by Dillner *et al.* [1990]. The optimal dilution of serum was found to be a 1:20 dilution, and was used for all subsequent ELISAs. Horseradish peroxidase conjugated rabbit-antihuman IgG and IgA were used at concentrations suggested by the manufacturers, as this dilution gave optimal performance. Microtiter plates were coated overnight at 4°C with 100 μl of the appropriate peptide antigen in 10mM carbonate-bicarbonate buffer, pH 9.6 (Appendix 2). The peptides were diluted to a concentration of 20μg/ml giving a coating quantity of 2μg/well. Unbound antigen was removed by washing with PBS in an ELISA plate washer (Sanofi, LP55, Diagnostics Pasteur), and free binding sites were blocked by the addition of 100μ M/PBS-T per well for 60 min at 37°C. Plates were washed 3x with PBS-T. Thereafter 100μl of serum sample (diluted 1:20 in 1% milk powder in PBS-T) was added to each well, and plates incubated at 37°C for 60 min. Wells were washed 3x with PBS-T before the addition of 100μl horseradish peroxidase conjugated rabbit-antihuman IgG (Dako,

Sweden), diluted 1:6000 in M/PBS-T. Plates were incubated for a further 60 min at 37 °C. The wells were again washed 3x before the addition of 100µl 1,2-phenylenediamine (Dako, Sweden) in 0.1M citric acid-phosphate buffer, pH 5 (diluted according to the manufactures instructions) with 0.006% hydrogen peroxide. Plates were left at room temperature in the dark. The colour reaction was stopped after 30 min by the addition of 100µl of 0.5M sulphuric acid and the absorbences read at 492nm (A_{492}) on an Anthos 2001 plate reader (Anthos Labtec Instruments, Austria), to determine the optical density values (OD). For each serum the difference in optical density (dOD) was calculated (mean OD of duplicate antigen-coated wells minus the mean OD of 2 wells coated with PBS-T buffer only). To control for plate-to-plate and day-to-day variability, positive and negative control sera (pooled positive adult and negative children's sera respectively), were included on each plate. Any variation of > 10% in the control sera on a plate, resulted in the results being discarded and the experiment repeated.

VLP ELISAs were performed as described by Rose *et al.* [1994a]. Antigen was diluted to 10µg/ml in cold PBS and then added to microtiter plates, using 100µl/well, and allowed to adsorb overnight at 4°C. Unbound VLPs were removed by washing twice with PBS and the plates blocked with 1% milk powder in PBS (M/PBS) (without tween) for 120 min at room temperature. After washing wells 3x with PBS, serum diluted 1:20 in 1% milk powder in PBS was added (100µl/well) to the microtiter plates and then left at room temperature for 120 min. Thereafter the procedure was as for the peptide ELISA. There was concern that non-specific "background" could be caused by antibodies to baculovirus or tissue culture proteins that co-purify with the VLPs, or by reactivity to cross–reactive epitopes generated on disrupted VLPs. To exclude non-specific values, the mean OD value of the serum on 2 wells coated with BPV-VLP was subtracted from the mean OD obtained from 2 wells coated with VLP-16. It was postulated that since both the BPV-VLP and the VLP-16 preparation had been prepared in the same way, they should have the same amount of co-purifying proteins and VLP breakdown products. These corrected results were designated VLP-16_{corr}.

2.2.4. Data analysis

Data was analysed by χ^2 test using Epi Info Version 5 (Centers for Disease Control, Epidemiology Program Office, Atlanta, Georgia USA). In all tests the significance level for assessing deviations from the tested hypothesis was P = 0.05. Data were examined using two cut-off points for seropositivity. This was achieved by calculating the mean and standard deviations (SD) of the absorbance (OD) values obtained from children's sera after reactivity with each antigen and eliminating those sera with values greater than mean plus 3 SD (outliers, who could be HPV-16 antibody positive). This was repeated (usually twice) until none of the remaining sera were excluded and the final mean value plus 2 SD (mean+2SD) taken as the cut-off value. Regression trendline analysis was by the least-squares method using Microsoft Excel (Microsoft Corp. Redmond, WA, USA).

2.3. RESULTS

2.3.1. Seroprevalence of antibodies in adults

The presence of IgG antibodies was measured in sera of 95 women with CIN (patients) and an equal number of age-matched female blood donors (controls) to HPV-16 and HPV-18 peptides and VLPs. Results using a cut-off, mean+2SD of the children's sera, are given in Table 2.I. The use of the BPV-VLPs to eliminate non specific background (VLP-16_{corr}) increased the specificity of the test, but may have reduced the sensitivity of the test by scoring some low positive sera, negative (Table 2.I.). However, there was little change in OR or significance levels when comparing the seropositivity to VLP-16 and the VLP-$16_{corr.}$ values (patients vs controls).

Table 2.1. The incidence of IgG antibodies to papillomavirus antigens in CIN patients and age-matched controls.

	CIN patients (95) n (%)	Controls (95) n (%)	Odds Ratio	95% CI	P value*
E2-18	20 (21)	19 (20)	1.07	0.5-2.28	0.8574
E2-16	23 (24)	12 (13)	2.21	0.97-5.10	0.0039
L1-16	8 (8)	12 (13)	0.64	0.22-1.78	0.34443
VLP-16	43 (45)	19 (20)	3.31	1.66-6.65	0.0002
BPV-VLP	15 (16)	11 (12)	1.43	0.58-3.58	0.3984
VLP-16_{corr}	32 (34)	14 (15)	2.94	1.37-6.63	0.0022

* χ^2 test for difference between two categories, CI = confidence interval.

For each antigen the patients were compared to the controls to establish if there was any correlation between seropositivity and disease status. Seropositivity to VLP-16 was significantly associated with CIN (P = 0.0022) and to E2-16 the association appeared significant (P = 0.0039) but the CI bisected 1.0 so precluded this despite the *P* value. Antibodies to BPV-VLP, L1-16 and E2-18 were not significantly different in women with CIN and controls. For all antigens except E2-16 the OR did not change using either cut-off (mean+2SD or mean+3SD). Three-times as many CIN patients than controls were seropositive to the E2-16 peptide using the cut-off of mean+3SD (19% vs 6%; P = 0.008; OR = 3.47; CI = 1.22-10.34). There was no correlation between the seropositivity to the E2-16 peptide and VLP-16 in the same person. The seropositivity to the other antigens in the same individual also did not correlate. There were differences found in the percentage of patients with antibodies to VLP-16 and E2-16, when the patients were grouped according to the severity of their disease (Table 2.2). The women with CIN 1 (6) were grouped with the women with CIN 2 (15) as CIN1/2 (21), because both groups were small.

Table 2.2. The comparison of IgG antibodies to VLP-16_{corr} and E2-16 in women with CIN1/2 or CIN 3.

Antigen	CIN1/2 (n=21)		CIN 3 (n=74)		P value
VLP-16	3	14.3%	29	39.2%	0.03
E2-16	4	19%	19	25.6%	0.5

There were more women with IgG antibodies to both E2-16 and VLP-16 antigens in sera of patients with CIN 3 than in sera of patients with CIN 1/2 (Table 2.2), but the difference was only significant for antibodies to VLP-16 (P = 0.03).

2.3.2. The seroprevalence of antibodies in children

ELISAs of the 155 children's sera revealed that a significant number of children had antibodies to HPV-16 and HPV-18 antigens (Table 2.3.). Data is presented with two ELISA cut-off values, ie. mean+3 SD and mean+2 SD. The former values may result in some sera being scored as negative (presumably false negatives). However, even when using the higher cut-off value, a significant number of children were positive for antibodies to HPV-16 E2 and L1 as well as to HPV-18 E2 peptides. In contrast antibodies reacting with VLPs were infrequently detected in children's sera. Antibodies reactive with the E2-16 peptide were present in more than twice as many children's serum samples (44.5%), as samples with antibodies to the L1-16 peptide (20%). Seropositivity was not related to the gender of the child.

Table 2.3 The incidence of IgG antibodies to papillomavirus antigens in children

antigen	Number positive				
	mean+2 SD		n = 155	mean+3 SD	
	n	(%)		n	(%)
E2-18	29	(18.7)		23	(14.8)
E2-16	69	(44.5)		39	(25.1)
L1-16	31	(20)		28	(18.1)
VLP-16	7	(4.5)		5	(3.2)
BPV-VLP	8	(5.1)		6	(3.8)
$VLP\text{-}16_{corr}$	4	(2.5)		2	(1.3)

2.3.3. The correlation of HPV-16 seropositivity with age

Rates of seropositivity in adult women with CIN were analysed to assess the extent of variation (if any) according to age. Data was assessed for patients in 4 age groups: 24 aged 21-30 years; 45 aged 31-40 years; 18 aged 41-50 years and 8 older than 50 years and

compared with equal numbers of age-matched controls. Results in Table 2.4. show the number of positive patients (or controls) in each age bracket with antibodies to the E2-16, E2-18 or L1-16 peptides and to VLP-16 or BPV-VLP and VLP_{corr}.

Table 2.4. The distribution of IgG antibodies to HPV-derived peptides and VLPs in adults according to age.

	Age in years							
	21-30, n = 24		31-40, n =45		41-50, n = 18		> 50, n = 8	
	Patient	Control	Patient	Control	Patient	Control	Patient	Control
E2-18	3 (12)	3 (12)	11 (24)	9 (20)	5 (28)	5 (28)	1 (12)	3 (38)
E2-16	3 (12)	3 (12)	11 (24)	7 (16)	6 (33)	2 (11)	3 (38)	0
L1-16	4 (17)	3 (12)	3 (7)	7 (16)	1 (6)	2 (11)	0	0
VLP-16	16 (67)	7 (29)	20 (44)	8 (18)	5 (28)	3 (17)	2 (25)	1 (12)
BPV-VLP	3 (12)	4 (17)	3 (7)	4 (9)	3 (17)	2 (11)	2 (25)	1 (12)
$VLP\text{-}16_{corr}$	12 (50)	5 (21)	12 (27)	7 (16)	4 (22)	2 (11)	1 (12)	0

Values in parentheses are percentages

The E2-16 antibody prevalence increased significantly in the older age groups in CIN patients, but decreased with age in the control group (Fig.2.1a). There was a decreasing trend with decreasing age for the presence of antibodies in both groups to $VLP\text{-}16_{corr}$. The good degree of fit of the regression trendline (Fig.2.1a) was demonstrated by $R^2 = 0.96$ for the patients. For the controls the degree of fit of the trendline was not good ($R^2 = 0.64$).

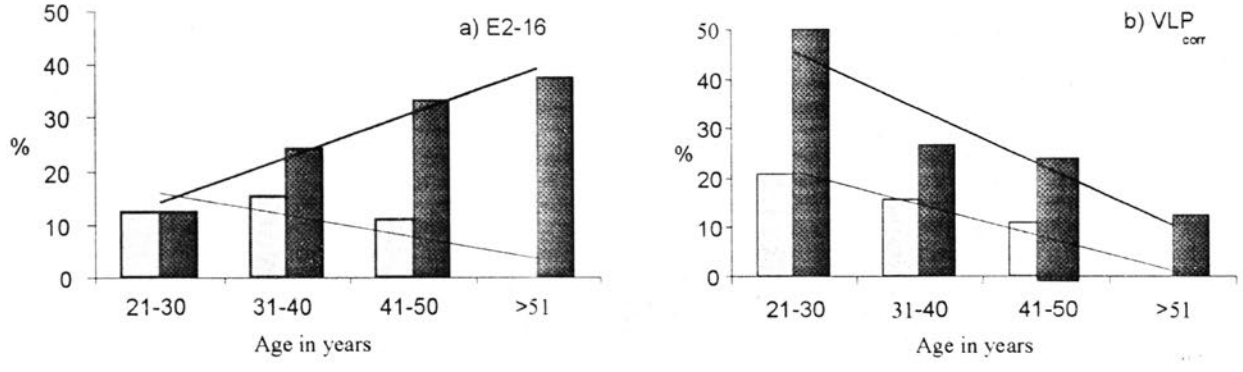

Fig. 2 1. The incidence of IgG antibodies to the HPV-16 E2 peptide (a) and to $VLP\text{-}16_{corr}$ (b) in CIN patients (black squares) and healthy controls (grey squares) according to age. Trendlines a) E2-16: patients (———) $R^2 = 0.96$, controls (·····) $R^2 = 0.64$;
b) $VLP\text{-}16_{corr}$: patients (——) $R^2 = 0.90$, controls (·····) $R^2 = 0.95$.

The prevalence of antibodies to E2-16 amongst patients 40 years and younger (14/69) was not significantly less than amongst patients older than 40 years with a relative risk (RR) of 1.71 (P = 0.14). The reverse was true for VLP-16 (or VLP-16_{corr}), in which antibody prevalence was highest in the 20-29 age group in both patients and controls and decreased significantly with age for both patients and controls (Fig. 2.1b). The regression trendlines (Fig.2.1b) demonstrated a good degree of fit for both patients (R^2 = 0.90) and controls (R^2 = 0.95). Prevalence to VLP-16 in the over 40 age group was lower (7/26) than in the under 40 age group (36/69) with RR= 1.94 (p=0.02). There was no significant association with age for the L1-16 peptide or the BPV-1 VLP antigens.

In the children's group the prevalence of antibodies to the E2-16 peptide decreased with age from 3 to 12 years (Fig. 2.2a).

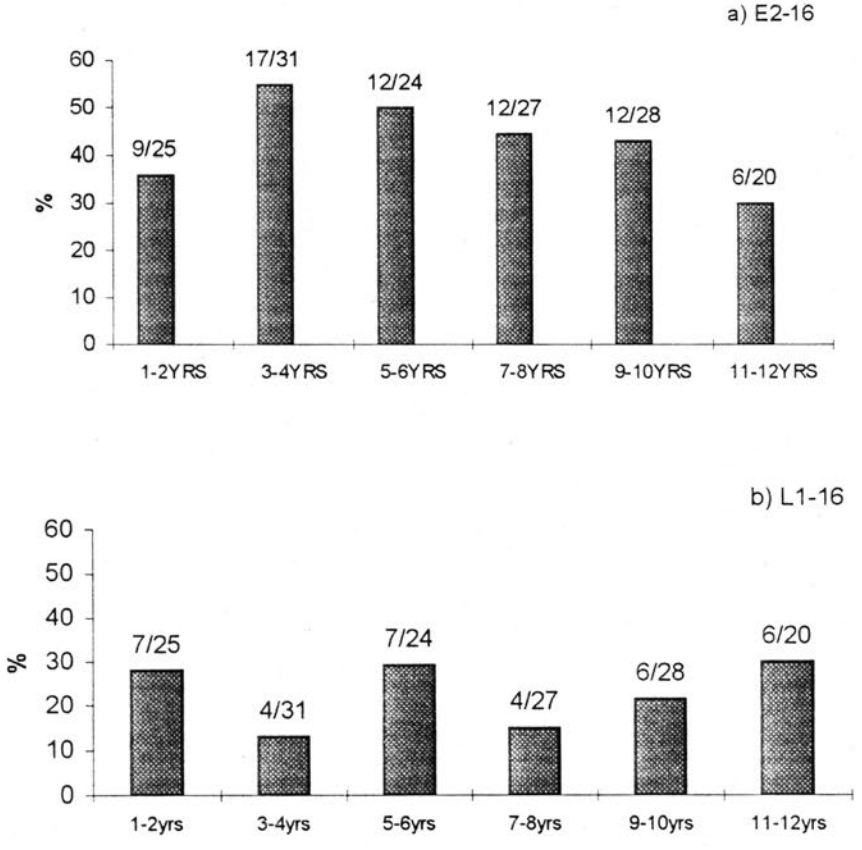

Fig 2.2. The distribution of IgG antibodies to HPV-16 antigens in children according to age. a) E2-16 and b) L1-16.

Fewer children were seropositive aged 1-2 years than 3-4 years. Seropositivity to the L1-16 peptide showed no association with age (Fig.2.2b). Amongst the children there were too few seropositive sera to look at the age distribution with the other antigens.

2.4. DISCUSSION

This study has shown a higher incidence of antibodies to E2-16 and VLP-16 in women with CIN than in female blood donors, with the seroprevalence of antibodies to VLP-16 correlating significantly with disease. This was the first serological HPV study to be done on women from South Africa and it was important to validate the results by comparison with similar studies elsewhere. The results were found to compare favourably with other such studies [Dillner *et al.*, 1994; Kirnbauer *et al.*, 1994; Nonnenmacher *et al.*, 1995]. The percentage seroprevalence found for the CIN patients and controls to VLP-16 was similar to that found by Chua *et al.* [1996] in a prospective study. However, the percentage of women with CIN who are seropositive to E2-16 or VLP-16 is lower than reported by Strickler *et al.* [1994]; Dillner *et al.* [1994] and Nonnenmacher *et al.* [1995]. This is probably because of the different distribution of HPV types found in women with cervical disease in South Africa [Williamson *et al.*, 1989] and a lower prevalence of HPV16 (46%) in women with CaCx than elsewhere [Williaimson *et al.*, 1994]. This indicates a possible greater effect that the other oncogenic HPV types, other than HPV-16, might have on cervical disease in South Africa. The prevalence of VLP-16 antibodies in the donor controls was double that reported in Spain, which unlike South Africa, is a country with a low incidence of cervical cancer [Nonnenmacher *et al.*, 1995]. Donors are a convenience sample that are commonly used to estimate seroprevalence in the general population [Strickler *et al.*, 1999], however nothing is known about their disease status and some could be infected with HPV-16.

Other serological studies have not reported the age distribution of positive sera from women with CIN [Dillner *et al.*, 1989; Nonnenmacher *et al.*, 1995; Dillner *et al.* 1994]. An age-dependant distribution of antibodies to certain peptide antigens in CaCx patients and normal controls was described by Dillner *et al.* [1994], but no statistically significant difference was seen with age for the E2 245-16 peptide used in that study, except an increased relative

risk in women over 50 years. Dillner *et al.* [1994] reported a peak in seroprevalence to certain peptides in those aged between 30 and 39 years, (245:16, E701 and 245:6) mainly for IgA, after which antibody seroprevalence declined. The present study found that the occurrence of antibodies to E2-16 increases with age in women with CIN whereas the converse is true in the age-matched blood donors. Age differences in seropositivity to E2-16 have been described in normal healthy people [Hamšiková *et al.*, 1994; 1998] comfirming that age should be taken into account when using the E2-16 peptide in the assessment of cervical disease. Unlike seropositivity to E2-16, the seropositivity to VLP-16 in the present study, decreased with age in women with CIN. Certain studies have found no relationship of age with seroprevalence to VLP-16 [Dillner *et al.*, 1996; Chua *et al.*, 1996], but recently Tachezy *et al.* [1999] have described a lower seropositivity to VLP-16, -18, and -33 as women with cervical disease increased in age. Therefore antibodies to VLP-16 (and VLP-18 and -33) may only be serological markers of disease in younger women and are unlikely to prove a reliable marker of disease in older women. In women with normal cytology the prevalence of HPV DNA has been shown to decrease with age [Melkert *et al.*, 1993; Gradilone *et al.*, 1996], which is reflected in the present observations of the decrease in the blood donor antibodies to E2-16 and VLP-16.

It is difficult to explain why the immune response to different HPV antigens in women with the same lesion should vary with age. The hormonal status of the women could influence the different antigen responses, as steroid hormones have been shown to increase HPV transcription [Pater *et al.*, 1994]. CIN in the younger women may be associated with a more recent productive viral infection that may result in more virus particles being produced and the subsequent induction of a good immune response. The older women may have had the lesion longer and are no longer shedding virus particles, but are still producing early HPV proteins. This hypothesis would be consistent with the integration of viral DNA in advanced disease, with the concomitant loss of late gene expression [zur Hausen, 1994]. It has been reported that antibodies to L1 are short lived unless there is a persistent infection [Wikström *et al.*, 1995] which implies that unless there is a productive HPV infection, L1 antibodies would decrease with time.

This was the first study reporting the prevalence of anti-E2-16 and E2-18 antibodies in children. There was a trend for the prevalence for antibodies to E2-16 to decrease with the age suggesting that children are infected early in life and therefore that the route of infection would be non-sexual. Some of the children may have been infected during birth. Cason *et al.* [1995] demonstrated high transmission rates from mother to infant, where HPV-16 DNA was detected in 83% of babies born of HPV-16 positive mothers, up to six months after birth. Other studies have reported similar transmission from mother to infant [Sedlacek *et al.*, 1989; Pakarain *et al.*, 1994; Puranen *et al.*, 1997].

As the control adults in this study had a lower seroprevalence of antibody to peptide antigens than did the children, it is probable that the infections in children are not persistent. Children with antibodies to HPV-16 L1, L2, E4, E6 and E7 proteins have been reported in other studies [Cason *et al.*, 1995; Muller *et al.*, 1995]. A similar high seroprevalence to the E2-16 peptide has been reported in children in Czechoslovakia [Hamšiková *et al.*, 1998]. Their study also described a decreasing trend of seroprevalence in children from 3 to 12 years. It would be important to determine if mucosal immunity persists and whether adults that have been infected as children have a successful immune response to re-infection with HPV-16 resulting in fewer persistent infections. This information would have significant implications for HPV vaccine strategies.

This study has demonstrated that few children have an IgG serological response to the antigens presented on VLP-16 and are more likely to have antibodies to E2-16. The reason for this is unclear but may indicate a lack of sensitivity in the detection of VLP-16 antibodies or a lack of induction of IgG to VLP-16 in children. Other reports on the low seroprevalence to VLP have been documented in children [Luxton *et al.*, 1997; Hamšiková *et al.*, 1998; af Giejersstam *et al.*, 1999]. Since HPV E2 proteins are expressed in the lower layers of the epithelium and the L1 proteins are produced in the upper layers of the epithelium [Pfister and Fuchs, 1987], there is more likely to be an immune response to the early proteins. Alternatively, most infections in children mucosal HPV by oncogenic HPV types are thought to be subclinical and non-productive, and in such infections the amount of L1 protein made may be reduced, although early proteins are expressed.

The possibility of the children's seropositivity being false is unlikely as the results obtained with adult sera are similar to those reported elsewhere [Dillner *et al.*, 1989; Nonnenmacher, 1995; Dillner, 1994; Chua *et al.*, 1996], confirming that the test was reliable. However the possibility of cross-reactivity with related antigens cannot be ruled out. The present study showed no correlation between the positive results obtained with the different HPV-16 antigens in the same person, which raises the question of whether the tests are specific for HPV-16, or whether there are a number of independent humoral reactions. Alternatively different antibody responses may be detected at different stages of HPV infection. Strickler *et al.* [1997] also reported no correlation between antibody responses to VLP-16 and HPV-16 E6 in women with CIN and suggested that there were at least two independent groups of humoral reactions.

There was no increase in seropositivity found to the E2-18 peptide in women with CIN compared with control women. This is consistent with results from a previous study which showed that HPV-18 DNA was rarely associated with cervical cancer in Cape Town [Williamson *et al.*, 1994], but was found in 3.5% of Cape Town women with normal cervical cytology [Ramesar *et al.*, 1996]. The L1-16 peptide was found to be poorly immunogenic in adults, in contrast to the results of Cason *et al.* [1992], using the same peptide, and in agreement with Le Cann *et al.* [1995]. The children showed the highest seropositivity to this peptide, which might be an indication of cross-reactivity with antigens of other HPV types.

In conclusion this study has shown that antibody responses to VLP-16 are significantly associated with CIN and to E2-16 approaching a significant level. However, these associations appeared age dependent in adults and therefore age has to be considered in the search for serological markers of HPV associated cervical disease. This study was conducted on relatively small numbers of sera and should be repeated with larger numbers and a wider range of cervical lesions should be examined to determine the relevance of age and seroprevalence to HPV antigens. The high incidence of E2-16 antibodies in children suggests that a high proportion of children may be infected with HPV-16 or a related virus,

with no apparent disease. HPV DNA isolation from children and further serological studies should help resolve some of the questions raised by these data. An extension of this study was necessary in order to the determine the prevalence of further HPV types in the South African population and to include in the study, the association of HPV types with CaCx.

CHAPTER 3. ANTIBODIES TO VIRUS-LIKE PARTICLES AS MARKERS OF CERVICAL DISEASE

Women with CIN and cervical cancer display a broadening of the humoral response to VLP epitopes

3.1. INTRODUCTION

The main HPV types associated with CaCx worldwide as described in Chapter 1.9, are HPV-16 (about 50%) followed by HPV-18 (14%), HPV-45 (8%) HPV-31 (5%) and HPV-33 (3%) [Bosch *et al.,* 1995]. In South Africa, HPV DNA studies done in Cape Town (Chapter 1.10.) and Durban indicate that HPV-16 is the predominant type associated with cervical disease [Williamson *et al.,* 1989; Williamson *et al.,* 1994; Cooper *et al.,* 1991]. We have at present, little other information pertaining to the southern African region with regards to the most prevalent types circulating in communities, or those associated with disease. Seroreactivity to HPV VLPs is reported to be a better marker for CaCx risk than HPV DNA determination [Nonnenmacher *et al.,* 1995 and 1996]. Populations with a high CaCx incidence (as in South Africa), are also reported to have a higher HPV-16 prevalence than those with a low CaCx incidence [Strickler *et al.,* 1999]. With HPV vaccines under development, it will be imperative to determine the prevalent types within a region and which of those are associated with cervical disease.

Seroreactivity to VLP-16 has been shown to be associated with the presence of HPV-16 DNA at the cervix [Kirnbauer *et al.,* 1994]. VLP-based ELISA tests, as described in Chapter 1.11.2, will detect serological evidence of past, present and persistent HPV infection [Kirnbauer *et al.,* 1994; Wikström *et al.,* 1995; Wideroff *et al.,* 1995]. It has been demonstrated too that antibodies to HPV-16 VLP (VLP-16) and VLP-18 and VLP-33 antibodies are markers of sexual behavior [Dillner *et al.,* 1996; Viscidi *et al.,* 1997; Olsen *et al.,* 1997]. In addition, antibodies to VLP-16 predict cervical disease [Dillner *et al.,* 1995; Nonnenmacher *et al.,* 1995; Carter *et al.,* 1996] and VLP-16, VLP-18 and VLP-33 seroresponses are significantly associated with CaCx [Wang *et al.,* 1997a].

In Chapter 2, using VLP-based ELISA, it was possible to detect IgG antibodies to HPV-16 VLPs in 45% of women with CIN and 19% of blood donor controls. This seroprevalence was found significantly associated with CIN. Work described in the present chapter was to extend this investigation to an evaluation of exposure to HPV types 16, 18, 31, 33, and 45 and to assess the relationship between seroreactivity to these HPV types and cervical cancer as well as CIN in South African women. To achieve this, sera from female blood donors (controls), matched in age with women with CIN, and women with CaCx were tested by VLP ELISA for antibodies to the five different HPV types. Following the observation of antibodies to HPV-16 and HPV-18 antigens in children's sera (Chapter 2.3.2.), these children were also assayed for antibodies to each of the five VLP types. Cervical biopsy material was available from the CIN and CaCx patients for HPV-16 DNA analysis. The VLP data was analysed to assess whether there was serological cross reactivity between HPV types.

An assessment of the seroresponses of all groups to the immunodominant region of the E7 protein of HPV-16, the E7 peptide (E7aa6-35), was also included in this study. Antibodies to this peptide, or similar E7-based peptides as described in Chapter 1.11.1.3, have been reported useful as serological markers of HPV-16-associated CaCx [Muller *et al.*, 1992; Sun *et al.*, 1994; Hamšiková *et al.*, 1994; Baayet *et al.*, 1995; Viladiu *et al.*, 1997].

3.2. MATERIALS AND METHODS

3.2.1. Serum and biopsy samples

Most of the sera from CIN patients, blood donors and children as described in Chapter 2, were included in this study. Some were omitted because of insufficient sample volume. In addition, serum and biopsy samples were drawn from 9 patients attending a colposcopy clinic and from patients receiving in patient care at Groote Schuur Hospital, Cape Town, during 1997. Histology of biopsies taken from all women at the same time as the serum sample, indicated that forty of these patients had CaCx (squamous cell carcinoma) and 95 had CIN (80 were CIN3, 11 were CIN2 and 4 were CIN 1). As age-matched controls, sera were obtained from 95 female, Western Province blood transfusion service donors. The number of CIN patients per age group were 5 aged 21-25, 17 aged 26-30 years, 28 aged 31-

35 years, 18 aged 36-40years, 12 aged 41-45 years, 6 aged 46-50 years and 9 older than 50 years (mean age 37). It was not possible to obtain age-matched blood donor controls for the CaCx group because of the advanced age of most of the members of this group (mean age 54 yrs). Cervical punch biopsies were obtained from all CIN and CaCx patients at the same time as the serum was collected and used for HPV DNA determinations. Children's (110) sera were all from patients at a private pathologist in Cape Town and were drawn for unrelated tests. The children were aged from 2 to 12 years.

3.2.2. HPV antigens for serology

3.2.2.1. E7 peptide

The E7aa6-35 synthetic peptide represents an epitope on the HPV-16 E7 protein (amino acids 6-35), as described by Müller *et al.* [1992]. The aa sequence was:
NH_2-PTLHEYMLDLQPEPTTDLYCYEQLNDSSEEE-OH
(Amino acid single letter designation, Appendix 1) The peptide was designated E7-16 and was synthesised by Genosys, USA.

3.2.2.2. Virus-like particles

VLPs were provided by Dr. Robert Rose, Rochester University, Rochester USA. VLPs were produced in insect cells [Rose *et al.*, 1993] from baculovirus-expressed recombinant L1 proteins of HPV-16, 18, 31, 33, and 45 and purified in CsCl gradients.

3.2.3. ELISAs

Sera (diluted 1:20) were tested for IgG antibodies by ELISA as described in Chapter 2.2.3, using 1μg VLP per well and 2μg/well of the E7-16 peptide. Each ELISA plate included positive and negative control sera as internal standards for the assays. To remove possible background seroreactivity sera were also tested against bovine papillomavirus type 1 VLPs (BPV-VLP) that were prepared in the same way (Chapter 2.2.3). In VLP ELISAs, for each serum sample the OD value obtained on BPV-VLP coated wells was subtracted from the mean of two values obtained on adjacent HPV-VLP coated wells (VLP_{corr}). The VLP_{corr}

value was used for all results. The CIN and control sera had previously been tested for reactivity to VLP-16, but were re-tested for VLP-16 reacitivity and assayed for reactivity to each of the other 4 VLP types at the same time, so each serum was tested on 5 BPV coated wells, 1 for each ELISA plate. For the peptide ELISA, the mean OD value of each serum obtained on 2 PBS-coated well was subtracted from the mean of two adjacent peptide-coated wells. If the results of the two values from which mean values were calculated were disparate the sample was rerun

3.2.4. ELISA cut-off values

ELISA cut-off values were determined for each VLP type and for the E7 peptide, using children's sera as described in Chapter 2.2.4. The respective cut-off values were for VLP-16 0.366, for VLP-18 0.98, for VLP-31 1.17, for VLP-33 1.93, for VLP-45 0.86 and for the E7 peptide 0.59. The difference in the cut-off values for the VLP ELISAs probably reflects differences in the quality of the relevant VLP preparations.

3.2.5. DNA extraction and HPV typing

DNA was extracted from biopsies as previously described [Williamson *et al.*, 1994]. Standard precautions were taken to prevent contamination with amplicon DNA and PCR artifacts. The quality of the DNA was tested by the amplification of the CCR5 gene [Michael *et al.*, 1997]. HPV-16 specific DNA was detected by amplification of the L1 gene as described by van den Brule *et al.* [1989]. All PCR analyses were performed by Patti Kay and Bruce Allen.

3.2.6. Data Analysis

Data was analysed as described in Chapter 2.2.4. Scatterplot analysis was achieved using Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

3.3. RESULTS

3.3.1. HPV Serology

3.3.1.1. E7-16 peptide-based serology

To determine the relationship of antibodies to an E7 peptide and cervical disease, the IgG responses to E7-16 were assayed for in the women with CaCx and CIN, blood donors (controls) and the children and the results are shown in Table 3.1.

Table 3. 1. The detection of IgG antibodies to the E7-16 peptide in CaCx and CIN patients, controls and children

antigen	CaCx patients		CIN patients		controls		children	
	n = 36	(%)	n = 95	(%)	n = 95	(%)	n = 110	(%)
E7-16	14	(39)	9	(9.5)	11	(11.6)	10	(9.1)

There was a significantly higher prevalence of antibodies in the CaCx patients (39%) compared with the CIN patients (9.5%), (P = 0.002). None of the 15 CIN 1 or CIN 2 patients were seropositive for E7-16, but 11.9% (9/80) of the CIN 3 patients were seropositive. The adults were examined for an age-related seroprevalence to E7-16 and no significant difference was found in the any of the groups.

DNA was extracted from biopsies of 24 of the 36 CaCx patients. Of the 24 biopsies, 13 were found HPV-16 positive (54.2%) by type specific PCR. Table 3.2. compares the E7-16 reactivities of CaCx patients with HPV-16 positive biopsies and CaCx patients with non-HPV-16 biopsies.

Table 3.2. The prevalence of E7-16 IgG antibodies in women with CaCx who were HPV-16 positive, and those not HPV-16 positive

	HPV-16 positive (13)		Non-HPV16 positive (11)		P Value
antigen	n	%	n	%	
E7-16	6	46.2	2	18	0.15

There were more than twice as many HPV-16 positive CaCx patients E7-16 seropositive compared with those patients not HPV-16 positive. The difference was however not significant (P = 0.15), possibly due to the small patient numbers. The small numbers of CIN patients E7-16 seropositive precluded any HPV DNA comparative analysis.

3.3.1.2. VLP-based ELISA

The distribution frequency of the ELISA OD values obtained for the reactivities of each of the children's sera to the 5 VLP types is shown in Fig. 3.1.

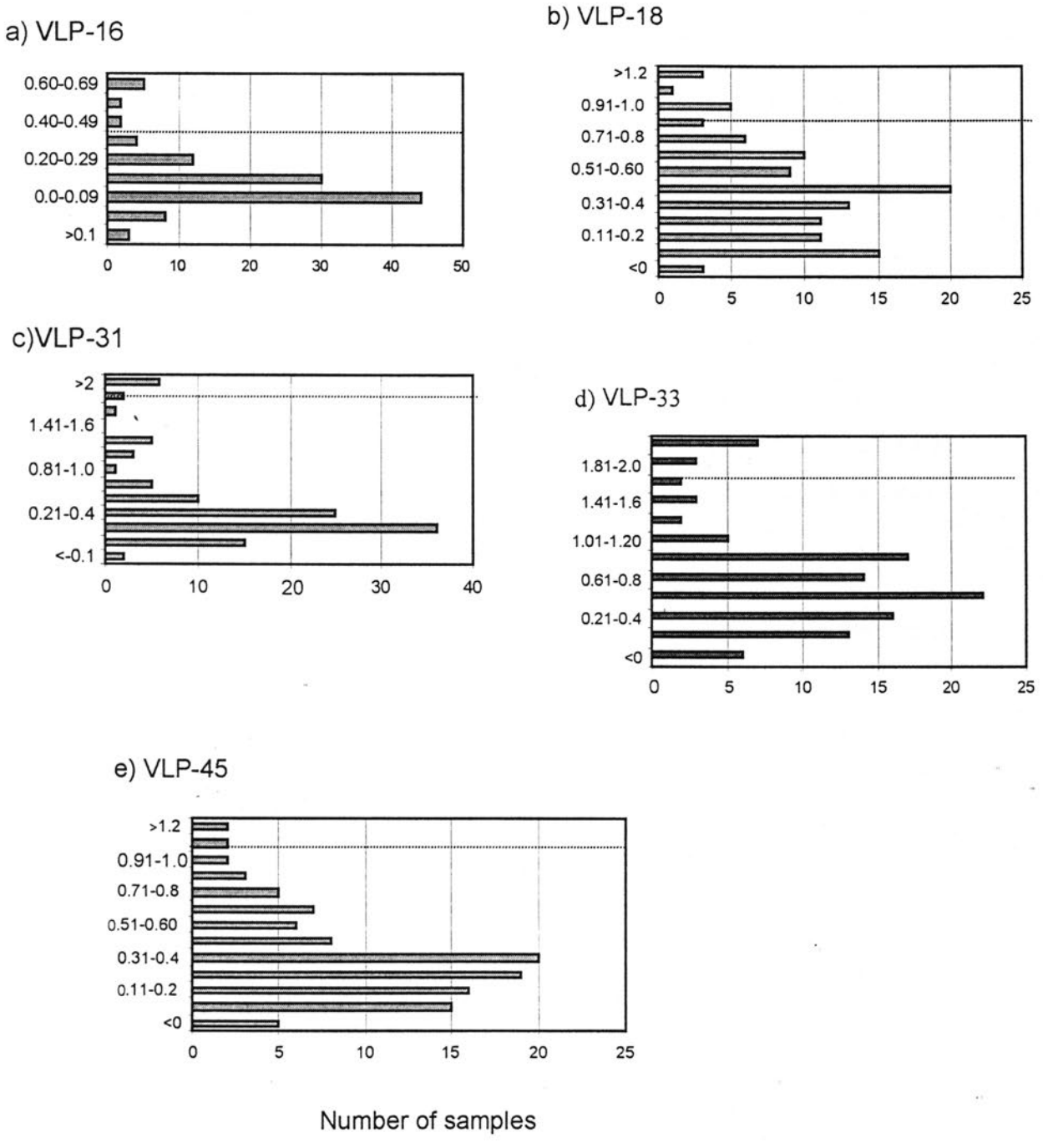

Fig. 3.1. The distribution frequencies of reactivities of the children's sera to the VLP types; a) 16, b) 18, c) 31, d) 33, and e) 45. The dotted lines on the graphs represent the cut-off values obtained for each VLP type. The OD values appear on the Y axis.

A very high cut-off value was obtained for the VLP-33 ELISA because of the large number of strongly reactive children's sera. The frequency chart (Fig. 3.1) for the children's reactivity to VLP-33 indicated a more realistic cut-off value to be 1.2. Results of assays for antibodies to VLP-33 were therefore compared using 2 cut-off values to establish the extent to which this would affect the incidence rates (Table 3.3).

Table 3.3. Detection of IgG antibodies in sera from children, controls and CIN and CaCx patients as measured in the VLP-33-based ELISA

Study group		Cut-off value 1.93	Cut-off value 1.2
Children	(n=110)	9 (8.2%)	17 (15.0%)
Controls	(n=95)	8 (8.4%)	30 (31.6%)
CIN patients	(n=95)	11 (11.6%)	34 (35.8%)
CaCx patients	(n=40)	6 (15.0%)	14 (35.0%)

In further reference to VLP-33, reactivity has been calculated using the higher cut-off value (1.93) as comparative incidence rates amongst the adult groups were not improved using a lower cut-off point.

The prevalence of IgG antibodies to the 5 different VLP types as measured in the sera of CaCx and CIN patients, controls and children is represented in Fig. 3.2

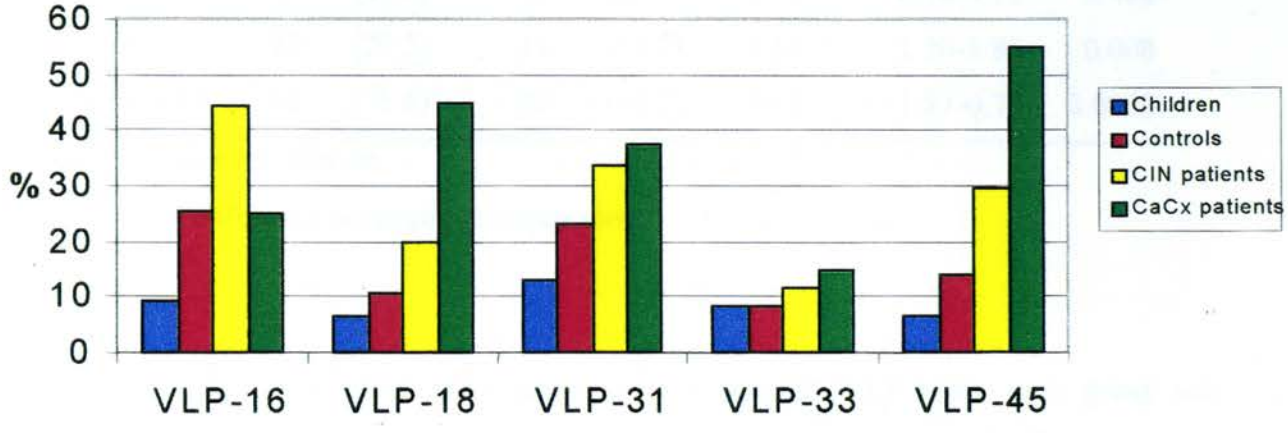

Fig. 3.2. The seroprevalence of antibodies to VLP-16, -18, -31, -33, and 45 as determined in CaCx and CIN patients, controls and children.

The frequency of antibody seropositivity to each of the five VLP types was higher in CIN patients than controls. With the exception of VLP-16, the antibody seroprevalence was even higher in women with CaCx than those with CIN. This was especially so for VLP-18 and VLP-45 (Fig. 3.2). In the case of VLP-16, the incidence of VLP-16 antibodies for those patients with CaCx, was no greater than that of the control group, whereas in CIN patients, antibodies to VLP-16 were the most prevalent of all the types. Statistical analysis showed a significant association with CIN (compared to controls) and seropositivity for antibodies to either VLP-16 (P = 0.006) or VLP-45 (P = 0.008) (Table 3.4.). When seroprevalence to any of the 5 VLP was combined, there was also a significant difference in prevalence to any VLP type between sera from women with CIN and control sera (P = 0.0002, OR=3.2).

TABLE 3.4 The seroprevalence of IgG antibodies to HPV-16, -18, -31, -33, -45 VLPs in CIN patients and age matched blood donor controls

	CIN patients		Controls		ODDS RATIO	95% CI	*P* value*
	n	%	n	%			
VLP-16	42	(44.2)	24	(25.3)	2.34	1.12-4.55	0.006
VLP-18	19	(20)	10	(10.5)	2.13	0.87-5.27	0.069
VLP-31	32	(33.7)	22	(23.2)	1.69	0.85-3.36	0.107
VLP-33	11	(11.6)	8	(8.4)	1.42	0.50-4.11	0.468
VLP-45	28	(29.5)	13	(13.7)	2.64	1.20-5.86	0.008
ANY VLP	68	(71.6)	42	(44.2)	3.18	1.83-6.73	0.0002

CI = confidence interval

* χ^2 test for difference between two categories.

Because so many individuals were seropositive to multiple VLP types, each group was examined with regard to their seronegative status, seropositivity to one VLP type and for multiple seropositivity (> 1 type). Results are shown in Fig 3.3.

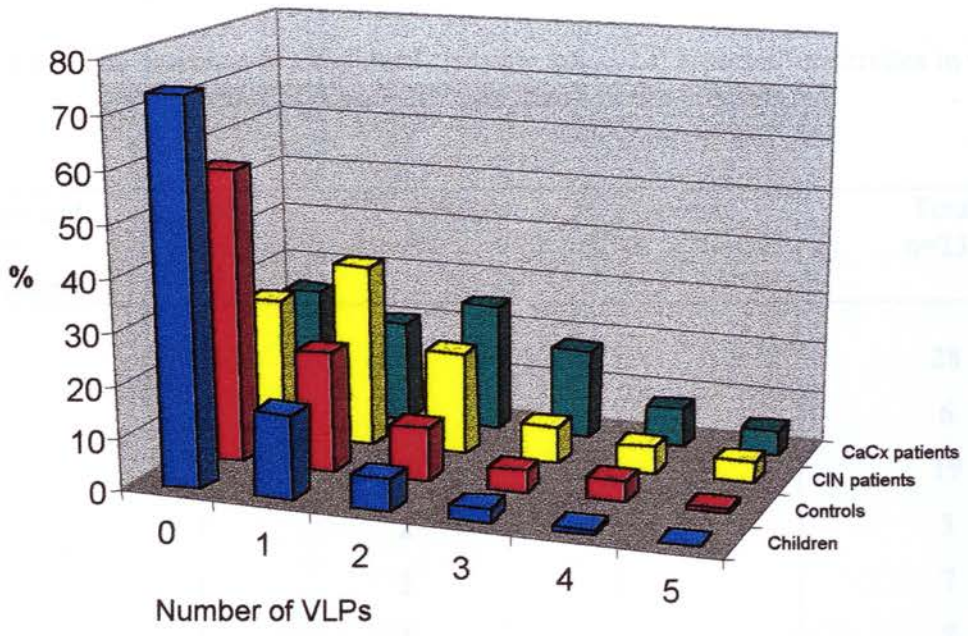

Fig. 3.3. The percentage of seronegative individuals and the percentage of individuals in each group (CaCx and CIN patients, controls and children) positive to 1-5 different VLP types.

The majority of children (72.7%) were found to be seronegative. Of the patients with cervical disease, 75% of CaCx patients and 71.6% of CIN patients were seropositive to at least one VLP type compared to 44.2% of the control group (P = 0.0002, CIN patients compared with controls). The majority of CIN patients had antibodies to either 1 or 2 types (54.7%) and 16.9% had antibodies to more than 2 VLP types. Of the CaCx patients, 27.5% had antibodies to >2 types. Table 3.5. represents the different positive VLP antibody combinations found in the sera of blood donor controls, or CIN or CaCx patient groups.

Antibodies most frequently found together in combination with one other in the same individual were to VLP-16 with VLP–31 and also to VLP-18 with VLP–45. Both combinations were found in as many individuals (34) but in 15 women both of these antibody combinations (VLP-16 with –31, and VLP-18 with –45) were found in the same person. There were more women with dual VLP-18 with VLP–45 antibody combinations (12) than those with dual VLP-16 with VLP–31 combinations (8).

Table 3.5. The presence of single and multiple anti-VLP type seropositivites in women with CaCx and CIN and blood donor controls.

VLP type and type combinations	Controls n=95	CIN patients n=95	CaCx patients n=40	Total n=230
16	10	17	1	28
18	4	1	1	6
31	6	10	3	19
33	1	2	0	3
45	1	3	3	7
16,18	1	1	0	2
16,18,31	0	0	1	1
16,18,31,33,45	1	4	2	7
16,18,31,45	2	4	2	8
16,18,33.45	0	0	1	1
16,18,45	1	3	1	5
16,31	3	5	0	8
16,31,33	1	2	0	3
16,31,33,45	2	1	0	3
16,31,45	1	1	2	4
16,33	1	1	0	2
16,45	1	3	0	4
18,31	0	1	1	2
18,31,45	0	0	1	1
18,33,45	1	0	1	2
18,45	0	5	7	12
31,33,45	0	1	0	1
31,33	2	0	1	3
31,45	3	3	2	8
TOTAL	42/95 (44%)	68/95 (72%)	30/40 (75%)	140/230 (61%)

3.3.2. The correlation of seroprevalence to VLPs with age in adults.

3.3.2.1. CIN patients and age-matched controls

The incidence of antibodies to each of the 5 VLP preparations was recorded for CIN patients and age-matched donor controls in two arbitrary age groups: one where the women were younger than 36 years and the other where they were older than 35 years (Table 3.6).

Table 3.6. The incidence of antibodies to each of 5 different VLP types and to >1 type in controls and CIN patients examined according to age <36 and >35 years.

VLP type	Controls (95)			CIN patients (95)		
	<36 yrs n = 50	>35 yrs n = 45	*P* value	<36 yrs n = 50	.>35yrs n = 45	*P* value
VLP-16	16 (32%)	8 (17%)	0.1	27 (54%)	15(33%)	0.04
VLP-18	5 (10%)	5 (11%)	0.86	13 (26%)	6(12%)	0.12
VLP-31	10 (20%)	12 (27%)	0.44	16 (32%)	16(36%)	0.71
VLP-33	3 (6%)	5 (11%)	0.37	3 (6%)	7 (16%)	0.11
VLP-45	4 (8%)	9 (20%)	0.09	15 (30%)	13 (29%)	0.9
> 1 VLP	8 (16%)	10 (22%)	0.44	22 (44%)	13 (29%)	0.12

In the control women, the incidence of antibodies to VLP-16 was twice as high in the younger women as in those over 35 years of age, but for each of the other VLP types, there was either no difference (VLP-18) or a somewhat higher incidence in the older women. No differences were significant. In the CIN patient group, the incidence of antibodies to VLP-16, –18 and –45 was lower in the older women and to VLP-31 and -33 higher in the older women. There were no significant difference between the incidence of antibodies to >1 VLP type in the older or younger control women. There was a significantly lower incidence of antibodies to VLP-16 in older women (>35 years) with CIN compared with younger women with CIN (P = 0.04), but no significant difference in the incidence of

antibodies to the other types in the two age groups. If the women with CIN in the two age groups were compared with regards seropositivity to >1 VLP type, more of those younger than 36 years, (44%, 22/50) were positive to >1 type than those older than 35 years, (29%, 13/45), but the difference was not significant ($P = 0.12$).

3.3.2.2. CaCx patients

Because of the advanced age of most of the CaCx patients, the incidence of antibodies in these women to each of the 5 VLP types was examined in those younger than 51 years (17) and those older than 50 years (23) (Table 3.7).

Table 3.7. The incidence of antibodies to 5 VLP types and to >1 type in CaCx patients younger than 51 and older than 50 years.

	CaCx patients <51 years (n=17)		CaCx patients > 50 years (n=23)		
VLP type	n	%	n	%	*P* value
VLP-16	4	24	6	26	0.57
VLP-18	5	29	13	56	0.09
VLP-31	7	41	8	35	0.67
VLP-33	1	6	5	22	0.17
VLP-45	2	29	17	74	0.00009
>1 VLP	6	36	16	70	0.03

In the women with CaCx, the incidence of antibodies was higher in the older women for all the VLP types except for VLP-31, where the incidence was slightly higher in the women younger than 51 years. There was a significantly greater incidence of antibodies to VLP-45 in the CaCx patients older than 50 years, compared with those younger than 51 years ($P = 0.00009$). Almost twice as many older women had antibodies to VLP-18 than those younger than 51 years, but this difference did not reach a level of significance ($P = 0.09$). If the CaCx patients in the two age groups were compared with regards seropositivity to >1 VLP type, there were significantly fewer women younger than 51 years (36%, 6/17) positive to >1 VLP type than older than 50 years (70% 16/23, $P = 0.03$).

3.3.3. The correlation of age and gender with the prevalence of VLP antibodies in children

The seropositivity to single and multiple VLP types as assayed in 95 children, with respect to age and gender is shown in Table 3.8. The age cut-off was arbitrary.

Table 3.8. The number of children displaying single and multiple seropositivities when assessed according to age and gender

VLP type	**Male** n=36	**Female** n=59	TOTAL* n= 95	**age <8 yrs** n=58	**age >7 yrs** n=37
16	0	4	**4**	1	3
18	2	0	**2**	1	1
31	1	5	**6**	2	4
33	4	1	**5**	5	0
45	0	1	**1**	0	1
16,18	0	1	**1**	1	0
16,18,31	0	1	**1**	1	0
16,31,45	1	0	**1**	1	0
16,18,31,45	0	1	**1**	1	0
18,33	0	1	**1**	1	0
18,45	0	1	**1**	1	0
31,33	1	0	**0**	1	0
31,33,45	0	1	**1**	0	1
total	9	17	**26**	16	10
%	25.0	28.8	**27.4**	27.6	27.0

* 15 children were excluded as no details of age or gender were available.

Most of the 27.4% of children who were seropositive, 69.2% (18/26), had antibodies to one VLP type, but 27% (7/26) of those who were seropositive had antibodies to multiple types. There was no significant difference between male and female children with regards to antibody prevalence and no apparent difference with regards to those who were younger

than 8 years or older than 7. However if the children's seroprevalence was assessed according to age groups (2 and 3, 4 and 5, 6 and 7, 8 and 9, 10 and 11years), a correlation of antibody prevalence with age was evident (Fig 3.4). There were **24** children aged 2 and 3 years, **16** aged 4 and 5 years, **18** aged 6 years and 7 years, **13** aged 8 and 9 years, **19** aged 10 years and 11 years and **3** aged 12 years.

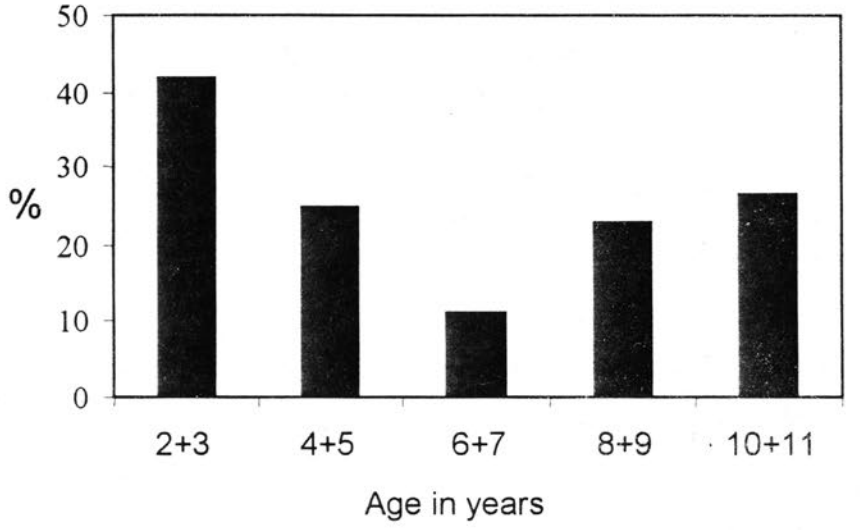

Fig. 3.4. The percentage of children with combined seroprevalence to 5 VLP types according to age groups. Children younger than 2 years of age were not included because of the possible presence of maternal antibodies. None of the 3 children age 12 years had antibodies to any VLP type.

There was a peak in the prevalence rate of VLP antibodies in the children at age 2 + 3 years and another at age 10 + 11 years.

3.3.4. DNA analysis

In order to determine the prevalence of HPV-16 infection in patients with cervical disease, DNA analysis was performed on cervical biopsies from 69 CIN and 24 CaCx patients (Table 3.9). All specimens were positive for the CCR5 gene, indicating adequate DNA for analysis. HPV-16 DNA was detected in 39/69 (56.5%) of CIN patients. There was no statistical difference between the numbers of CIN patients who were HPV-16 DNA positive as well as VLP-16 antibody positive (22/39), and the CIN patients who were HPV-16 DNA positive and seronegative (17/39) (P = 0.2). Amongst the CIN patients, the average age of those positive for both HPV-16 DNA and VLP-16 antibodies was 34 years,

whereas those who were DNA positive and VLP-16 seronegative had an average age of 52 years. Women with CIN with antibodies to VLP-16 who were negative for HPV 16 DNA had an average age of 37 years.

TABLE 3.9 Results of the HPV-16 DNA analysis of biopsies from women with CIN and CaCx and comparison to their VLP-16 antibody status

		HPV-16 DNA positive	HPV-16 DNA negative	Total
CIN	VLP-16 seropositive	22/69	9/69	31/69 (44.9%)
	VLP-16 seronegative	17/69	21/69	38/69 (55.1%)
	Total	39/69 (56.5%)	30/69 (43.5%)	
CaCx	VLP-16 seropositive	2/24	4/24	6/24 (25%)
	VLP-16 seronegative	11/24	7/24	18/24 (75%)
	Total	13/24 (54.1%)	11/24 (45.8%)	

HPV-16 DNA was detected in 54% (13/24) of CaCx patients. Only 2/13 (15%) of the CaCx patients who were HPV-16 DNA positive were also VLP-16 seropositive. Of the 11 women with CaCx who were HPV-16 DNA negative, four (31%) were VLP-16 seropositive.

3.3.5. Scatterplot analysis of seroreactivities to VLPs

The high incidence of seropositivity to multiple VLP types raised the possibility of serological cross-reactivity between the different VLP types. Therefore, scatterplot analysis of OD values obtained for each serum against each VLP type was compared with OD values obtained with the 4 other types. In adults, a weak correlation was found between seropositivity to VLP-18 and VLP-45 (Fig 3.5). For all other VLP types the R^2 value was < 0.3 when compared with one another.

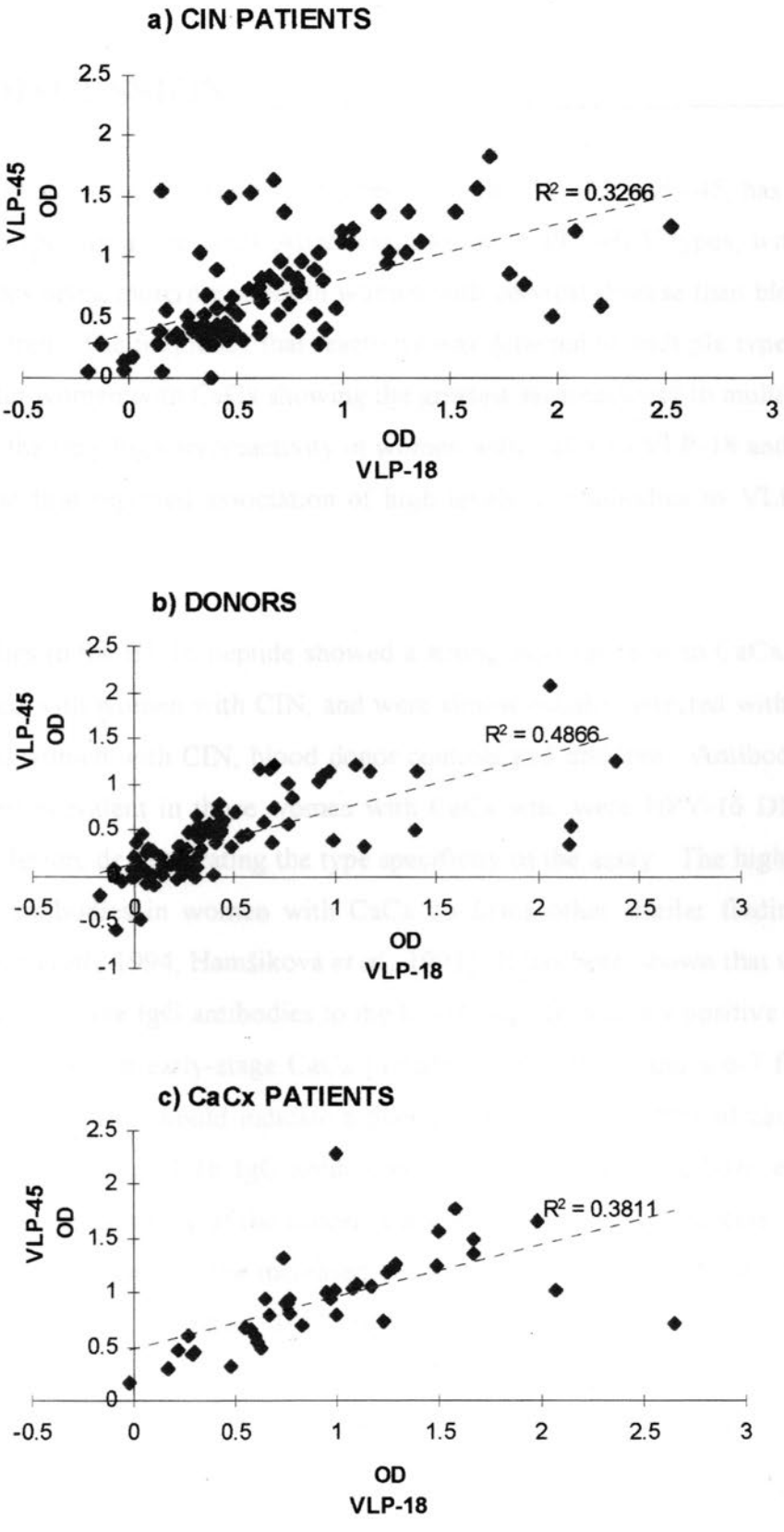

Fig 3.5. The comparison by scatterplot analysis of the seroreactivity to VLP-18 and VLP-45 in a) CIN patients, b) controls and c) CaCx patients. R = correlation coefficient.

3.4. DISCUSSION

The study of seroreactivity to VLP types –16, -18, -31, -33, and –45, has indicated the high level of exposure in the South African population to all 5 HPV types, with antibodies to all VLP types being more prevalent in women with cervical disease than blood donor controls and children. The frequency that reactivity was detected to multiple types was noteworthy, with older women with CaCx showing the greatest seroreactivity to multiple types. Of note too was the very high seroreactivity in women with CaCx to VLP-18 and VLP-45 with this being the first reported association of high levels of antibodies to VLP-45 with cervical disease.

Antibodies to the E7-16 peptide showed a strong association with CaCx ($P = 0.002$) when compared with women with CIN, and were almost equally detected with low incidence (9-11%), in women with CIN, blood donor controls and children. Antibodies to E7-16 were also most prevalent in those women with CaCx who were HPV-16 DNA positive at the cervical lesion, demonstrating the type specificity of the assay. The high prevalence of E7-16 IgG antibodies in women with CaCx confirms other similar findings [Muller *et al.*, 1992; Sun *et al.*, 1994; Hamšiková *et al.*, 1994]. It has been shown that up to 50% of CaCx patients may have IgG antibodies to the E7-16 peptide and this positive serology is related to poor survival in early-stage CaCx [Viladiu *et al.*, 1997], and a 6-7 fold increase in the risk of death. This would indicate a poor prognosis for the 39% of cancer patients in the present study with E7-16 IgG antibodies. Seroprevalence to E7-16 reportedly also rises with increasing severity of the cancer [Baay *et al.*, 1997]. The increase in E7 antibodies in CaCx patients relates to the increased and constant expression of the E7 protein in cancer cells [Smotkin and Wettstein, 1987; Stoler *et al.*, 1992].

In the present study 54% of women with CaCx were HPV-16 DNA positive, and of these, 46.2% were both seropositive to E7-16 and positive for HPV-16 DNA. This was not significantly higher than those who were not HPV-16 DNA positive (18%, $P = 0.15$). A negative correlation was found between E7-16 antibodies and those to VLP-16 amongst CaCx patients, with a higher seroprevalence rate for E7-16 than for VLP-16. This is consistent with the decreased expression in the L1 gene in cervical cancer cells and a concomitant increase in the expression of the E7 gene [Stoler *et al.*, 1992].

In the VLP ELISAs disease association was found for seropositivity to VLP-16 ($P = 0.006$) and VLP-45 ($P = 0.008$) in the CIN patients, compared with the blood donor controls. There was also disease association with the combined seropositivity to any of the five VLP types tested ($P = 0.0002$, OR=3.2). An association between VLP-16 antibodies and cervical disease is consistent with results from other studies worldwide [Chua *et al.*, 1996; Kirnbauer *et al.*, 1994; Dillner *et al.*, 1995; Wideroff *et al.*, 1995; Wang *et al.*, 1997; Tachezy *et al.*, 1999]. The present study found an elevated presence of antibodies in CaCx patients, compared with the CIN patients, for all VLP types except for the seropositivity to VLP-16. A lower seroprevalence to VLP-16 antibodies in CaCx patients compared to CIN patients confirms other findings [Nonnenmacher *et al.*, 1995], with a decrease in the VLP-16 antibody seroprevalence rate with the advancing stage of the invasive cancer [Park *et al.*, 1998].

As far as it can be ascertained, this is the first report of the association of CIN and antibodies to VLP-45. The implied disease association between VLP-45 antibodies and CaCx was unexpected as a previous study found that the prevalence of HPV-45 DNA was low (1.5%) in CaCx in Cape Town [Williamson *et al.*, 1994]. However, Bosch *et al.* [1995] has reported that 12.4% of CaCx from regions in central and northern Africa were associated with HPV-45 and the findings of this study and also that in Chapter 4, indicate an high prevalence of HPV-45 infection in southern Africa. It is difficult to reconcile the low HPV-45 DNA [Williamson *et al.*, 1994] prevalence with the large number of women with cervical disease in the present study with anti-VLP-45 antibodies. This will have to be clarified with HPV-45-specific DNA analysis on cervical material from women with cervical disease.

In the present study, seroprevalence to VLP-31 and VLP-33 showed no disease association. However more women with cervical disease had antibodies to VLP-31 and VLP-33 than controls and relatively more CaCx patients than CIN patients had antibodies. The increased prevalence of VLP-31 and VLP-33 antibodies in women with cervical disease could be of some significance but this remains to be determined. The seroprevalence amongst children to VLP-33 did not change relative to the adult groups, despite adjustment with two cut-off levels. The fact that no significant disease association ($P = 0.069$) was found between controls and CIN patients and the seropositivity to VLP-18, was surprising. This may be

due to the high prevalence of HPV-18 (detected by DNA analysis) amongst the normal population in South Africa [Ramesar *et al.*, 1996]. HPV serology would be expected to have the strongest predictive value for HPV associated disease in populations with a low HPV prevalence [Wang *et al.*, 1997a].

Most children had low seropositivity to the individual VLP types. However if seropositivity to the 5 VLP types were combined, the number of children seropositive to at least 1 type was 27.4%. This is a relatively large number of children who have been exposed to oncogenic HPV types. Transmission of HPV from a mother to her newborn child has been described [Cason *et al.*, 1995; Puranen *et al.*, 1997] and may account for some of these observations. The children's seroprevalence was higher at the age of 3 years and lower at age 6 years, with a further peak of reactivity around 10-11 years. Hamšiková *et al.* [1998] described seroprevalence in children to VLP-16, -18 and –33, with a similar decreasing trend in positivity from children younger than 6 years to those of 12 years, but no prevalence peak at 10 years, as in the present study. The higher seroprevalence in the younger children could indicate early infection (possibly from their mother), which is resolved with the dropping off of antibody levels, to increase again with the onset of sexual activity. There was no significant difference in seroprevalence between male and female children.

The previous chapter reported a decrease in seropositivity to VLP-16 with age. The present study compared the HPV-16 DNA status with serology. There was little correlation between positive HPV-16 DNA status and seropositivity to VLP-16, especially in older women. Nonnenmacher *et al.* [1996], Wang *et al.* [1997a], Carter *et al.* [1996] and Park *et al.* [1998] described a similar lack of correlation between serology and HPV-16 DNA status. In the present study, the average age in the CIN patient group of those HPV-16 DNA and VLP-16 antibody positive was 34 years, whereas those who were DNA positive and VLP-16 antibody negative had an average age of 52 years. Also, most of CaCx patients (11/13) who were HPV-16 DNA positive were VLP-16 antibody negative (average age 52 years). This suggests that antibodies to HPV-16 VLP antibodies may decline with time in women with high-grade lesions and that in most women with HPV-16 associated cancers VLP-16 antibodies were below the level detected by the ELISA. In cervical cancers there is no active virus production and therefore no detectable L1 expression [Bohm *et al.*, 1993]. The lack of L1 to stimulate the immune system in lesions may lead to

a drop in antibody titres. This relates to the observation in this study of the uncommon occurrence of antibodies to E7-16 and VLP-16 together in women with CaCX. However, Carter *et al.* [1996] found no reduction in VLP-16 antibody titres in college students followed up for four years after seroconversion. No change in HPV-16 VLP seropositivity was found in women between their first and second pregnancy [af Geijersstam *et al,* 1998]. In the present study the women were much older and the initial HPV infections could have occurred as much as 30 years earlier. A similar relationship between the presence of antibodies to VLP-16 and age in women with cervical disease has been described by Tachezy *et al.* [1999], who noted the same decrease in prevalence rate with an increase in age for antibodies to VLP-18 and VLP-33. In the present study the prevalence of antibodies to VLP-18 was lower in older women with CIN but higher in older women with CaCx. For antibodies to VLP-33, seroprevalence was higher in older women both with CIN and CaCx. The gradual decline in HPV-16 antibodies over time does not explain the high seroprevalence in the CaCx and CIN patients to certain VLP types. High levels of antibodies to the VLP-18, -31, -33 and 45 implies a more recent cervical exposure to these VLP types, or concurrent productive infection at a site other than the cervix. The immune response to VLP-16 did appear to differ in the women with CIN and CaCx as compared with the other VLP types, in that for antibodies to VLP-16 the seroprevalence rate was significantly lower with decreasing age. It has been postulated that HPV-16 may be less immunogenic than the other genital HPV types [R. Rose, personal communication], not inducing as great an immune response.

In the present study, seropositivity to multiple types was common and substantiated by a recent, similar report in Czech women [Tachezy *et al.*, 1999]. Indeed, more then half (75/140, 53.6%) the women, who were HPV VLP seroreactive were positive to more than one VLP type. The CIN and especially the CaCx patients were more likely to be seropositive to more than one VLP type compared with the controls and children. The total number of CIN patients seropositive to any of the 5 VLP types compared with the blood donor controls was significantly higher ($P = 0.0002$). The indication is however, that HPV infection is common in the general population and therefore *per se* does not predict progression to disease. Indeed, 27.4% of the all the children were also positive to at least one VLP type.

Significantly more of the CaCx patients also had antibodies to multiple VLP types, especially if over 50 years ($P = 0.03$) and more CIN patients younger than 36 years (44%) compared with older than 35 years (29%). This implies that these women are more susceptible to infection with multiple HPV types, are exposed to more types, or are more likely to have persistent infection, or are more likely to mount a measurable antibody response. Multiple seropositivity to VLPs could be related to sexual behaviour, as it has been reported that women with multiple seroprevalence have an elevated OR, related to a greater number of life-time sexual partners [L.Wideroff, personal communication]. This elevated OR can be reduced if the number of sexual partners are taken into account [Dillner *et al.*, 1996]. This implies that multiple seropositivity may be related to sexual behaviour and that seroreactivity to a number of VLP types could be the result of multiple HPV infections over time. This could explain why more of the younger women with CIN were seropositive to multiple VLP types. The number of sexual partners of the women under study was not known however, this factor should be considered in further studies.

Multiple sexual partners would be a less likely cause of multiple seropositivity in the women with CaCx who were over 50 years old. Multiple seropositivity could also be related to an altered immune response. In accordance with the immuno-dominance theory, the number of epitopes involved in an immune response could increase with repeated antigenic stimulation [Wang *et al.*, 1997b]. Early infections could stimulate a single dominant epitope, but upon prolonged antigenic exposure cross-reactive antibodies to minor epitopes could be introduced. It has been proposed that the one immunodominant, type-specific epitope on VLPs could be increased to include some cross-reactive epitopes [Wang *et al.*, 1997b]. In support of this theory, Hamšiková *et al.* [1994] reported a broader reactivity in CaCa patients to a number of HPV-16 E7 peptides than in controls. In the present study, the combinations of antibodies found most in individuals were to VLP-18 with VLP-45 and to VLP-16 with VLP-33. This corresponded with their phylogenetic relatedness and these closely related types would be more likely to have cross-reacting epitopes. Monoclonal antibodies have identified cross-reacting surface conformational epitopes on VLP-16 and VLP18, which are shared by their closely related HPV types [Christensen *et al.*, 1996]. More then half (57/98, 58.2%) of the women with cervical disease, who were HPV VLP seroreactive were positive to >1 VLP type and 69.6% of the CaCx patients who were older than 50 years were positive to >1 VLP type. This could indicate a broadening of the antibody response in the women with CaCx to react to multiple

cross-reactive epitopes. Individuals also showed seropositivity to different combinations of VLPs, indicating that if there is a broadening of the epitopes recognised, there must be different epitopes recognised in different women. Strickler *et al.* [1997] reported that several HPV antibodies may be related to CIN and that the immune response could be divided into at least two distinct groups. Whether all these findings are of significance in the development of HPV associated disease is unknown and further research is required in this regard.

This study was not able to confirm whether multiple seroprevalence was an indication of cross-reactivity between HPV types 16. 18, 31, 33, and 45, or whether it indicated infection with multiple types. There was probably a combination of these two possibilities. This will only be confirmed in longitudinal studies assessing the relationship between multiple viral exposure and seroprevalence. More than half the women (53.6%) and 30.8% of the children, who were HPV VLP seroreactive were positive to greater than one type. Seropositivity to multiple HPV types implies that infections by these types are in addition to that by HPV-16 and that infection by one type is not protective of infection by other types. It has been recently shown that there is no increased risk of CaCx among women seropositive to VLP-16 in combination with VLP-11 or -6 or -18 or –33 [Loustarinen *et al.*, 1999]. The latter study described an antagonistic interaction between these dual infections, which could be explained by cross protective HPV-specific cell-mediated immunity.

Knowledge of the HPV status of a population is necessary for the design of preventative measures. This study has demonstrated IgG antibodies to all five VLP types in the sera of groups assessed and antibodies to VLP-16 and VLP-45 to be associated with CIN. CaCx patients showed high seroprevalence levels to VLP-18, -31 and –45 and to E7-16. Whether the seroprevalence of HPV types 18, 31 and 45 is associated with the high incidence of CaCx in South Africa remains to be evaluated. This study emphasises the need for a multivalent HPV vaccine in the southern African region, as although there might be some serological cross reactivity, there does not appear to be appreciable cross protection between VLP types, with many women having antibodies to multiple types. Further studies of the HPV prevalence in different South African population groups could assist in confirming which are the most prevalent HPV types in the region and those associated with cervical disease.

CHAPTER 4. SERORESPONSES TO VIRUS-LIKE PARTICLES OF HUMAN PAPILLOMAVIRUS TYPES 16, 18, 31, 33 AND 45 IN SAN PEOPLE OF SOUTHERN AFRICA

4.1. INTRODUCTION

It is estimated that 6% of the 9 million cases of cancer recorded worldwide each year could be attributed to HPV infection, with women in developing countries at particularly high risk of HPV-associated cancers [IARC/WHO, 1999]. Like South Africa, Namibia have recorded very high prevalence rates for cervical HPV infection and cervical intraepithelial neoplasia (CIN) [Bloch *et al.*, 1988]. However, at present there is little information regarding the most prevalent HPV types, or those associated with cervical disease, in this region of southern African and no HPV typing has been performed on cervical specimens from Namibian women. In 1989, at the end of the Angolan war, a group of San people, also known as bushmen, were translocated from Omega in northern Namibia and were settled at Schmidtsdrift, in the Northern Cape province of South Africa and this community now consists of about 4,500 people [Gaobepe *et al.*, 1995]. These San people are descendents of the original indigenous hunter–gatherer people of southern Africa (Deacon, 1992) whereas the Negroid population, which now dominate the region, only migrated there a few hundred years ago (du Toit *et al.*, 1990).

As with many of the African communities, sera were available from the San people but no cervical specimens for DNA analysis. Therefore this study proposed to use HPV VLP serology as described in Chapter 3 to determine the exposure of the San people to the same 5 oncogenic HPV types. Seroreactivity to HPV VLP is reported to be a better marker for CaCx risk than HPV DNA (Nonnenmacher *et al.*, 1995; 1996]. These data would supplement the HPV seroprevalence rates determined in the Cape Town based study (Chapter 3) and be helpful in providing the background information needed to design appropriate HPV vaccines for specific communities.

4.2. MATERIALS AND METHODS

4.2.1. Serum samples

Sera from San volunteers from Schmidtsdrift were originally obtained in 1993 for a baseline epidemiological study and controlled immunisation programme with two recombinant hepatitis B vaccines [Gaobepe *et al.*, 1995]. At that time families were informed of the purpose of the study and volunteers were included in the study after signed consent from the families. Of the original sera collected, only 244 specimens remained for this study. These sera were derived from 115 children aged between 2 and 12 years, 17 teenagers, and 112 adults aged 20 to 83 years. The majority were female (61 children, 11 teenagers and 101 adults). The average age of the children was 8 years (female children average age 8.3 years and male children 7.6 years). The average age of the 101 women was 38 years. Of the males, 54 were children, 6 teenagers and 11 adults (average male adult age 41.6years).

4.2.2. Antigen

VLPs were obtained from Dr. Robert Rose, Rochester University USA, and were produced in insect cells [Rose *et al.*, 1993], from baculovirus-expressed recombinant L1 proteins of HPV-16, -18, -31, -33, and -45 and purified in CsCl gradients.

4.2.3. ELISA

San sera were tested for antibodies at a 1:20 dilution by ELISA, using a VLP concentration of 10μg/ml, as described in Chapter 2.2.3. Each serum was tested on 2 HPV-VLP coated wells and 1 adjacent BPV-VLP coated well. For each serum the OD value obtained on BPV-VLP coated well was subtracted from the mean of two values obtained on the HPV-VLP coated wells, giving a corrected OD value. For each VLP type the cut-off value was calculated from readings obtained using the San children's sera. The mean corrected OD values plus two standard deviations (mean + 2SD), after the elimination of outliers as described in Chapter 2.2.4, constituted the final cut-off value.

4.2.4. Data Analysis.

Data were statistically analysed as described in Chapter 2.2.4.

4.3. RESULTS

In ELISA the San peoples' sera gave a high level of background reactivity to VLPs, much higher than that of Cape Town blood donors and children (Chapter 3). It was not known whether this was due to antibodies to insect proteins in the San sera or to the storage of the serum. This different background level of ELISA reactivity emphasised the importance of the consideration of different cut points for different population groups and to include children from the same population group to calculate cut-off values. The San people were tested for their seroresponses to VLP-16, -18, -31, -33 and –45 and the prevalence of serum antibodies found is presented in Table 4. I.

Table 4.1. Seroprevalence of IgG antibodies to VLP-16, VLP-18, VLP-31, VLP-33 and VLP-45 amongst San people

	Number seropositive (%)		
VLP type	Children n=115	Teenagers n=17	Adults n=112
VLP-16	7 (6.1)	2 (11.8)	18 (16.1)
VLP-18	8 (7.0)	2 (11.8)	19 (17.0)
VLP-31	7 (6.1)	1 (5.9)	15 (13.4)
VLP-33	9 (7.8)	0	20 (17.9)
VLP-45	7 (6.1)	0	24 (21.4)

The seroprevalence amongst the children was low (<8%) for all the VLP types but 2-3 times higher amongst the San adults. Of the 26 children who were seropositive, 9 were positive to >1 VLP type (Table 4.2) and all but 2 of the 9 multiple seropositivities (Table 4.2) were in the male group. A higher percentage of the children < 8 years of age were seropositive (28%) than those > 7 years of age (18%) ($P = 0.05$) (Table 4.2).

A significantly greater percentage of the male children (33%) were seropositive than female children (13%) ($P = 0.0007$).

Table 4.2. Seropositivity in San children to a single VLP type or combinations of VLP types according to age and gender

	Number of seropositivities			
VLP types	Male (n=54)	Female (n=61)	Age<8 yrs (n=46)	Age>7 yrs (n=69)
16	3	2	4	1
18	1	1	0	2
31	1	2	0	3
33	5	1	3	3
45	1	0	1	0
16, 18	1	0	1	0
16, 45	1	0	0	1
18, 31, 33, 45	1	0	0	1
18, 31, 45	1	0	0	1
18, 45	2	1	2	1
31, 33	1	1	2	0
TOTAL	18/54 (33%)	8/61 (13%)	13/46 (28%)	13/69 (18%)

The seroprevalence of the San women to single and multiple VLP types is presented in Table 4.3. Of the seropositive women, almost half had antibodies to a single VLP, whereas the rest were seropositive to multiple VLP. Seropositive women were assessed in two age groups, those younger than 36 or older than 35 years of age, with regard to seropositivity to one or combinations of VLP types (Table 4.3). A significantly greater percentage of the older women (31/50, 62%) were positive as compared to the younger women (20/51, 39%) ($P = 0.02$). Five of the eleven men were seropositive: 2 were positive for HPV-31, one was positive for HPV-18, one was positive for HPV-33 and one positive for HPV-45 antibodies.

Table 4.3. Seropositivity in San women to single a VLP type or combinations of VLP types according to age.

VLP types	Number of seropositivities		
	age<36yrs n=51	age>35yrs n=50	TOTAL n=101
16	2	3	5
18	2	3	5
31	3	1	4
33	2	5	7
45	1	4	5
16, 18 ,31 ,45	1	0	1
16, 18, 33, 45	1	1	2
16, 18, 45	1	2	3
16, 31	2	1	3
16, 31, 33	0	1	1
16, 33	0	1	1
18, 31, 33, 45	1	0	1
18, 33, 45	0	1	1
18, 45	2	4	6
33, 45	2	1	3
31, 33	0	2	2
31, 45	0	1	1
TOTAL	20/51 (39%)	31/50 (62%)	51/101(50.5%)

The occurrence of seropositivity to increasing numbers of VLP types was assessed in children, teenagers and adults and the results are shown in Fig 4.1. The majority of the children (77.4 %) were seronegative, but of those that did show seroreactivity, most reacted with only one of the VLP types. In contrast, the presence of antibodies to 2, 3, or 4 different VLP types was more evident in the adults (22.7%). Almost half of the women had antibodies to only one VLP type, indicating a type specific response. This supports the probability of multiple infections rather than cross reactivity.

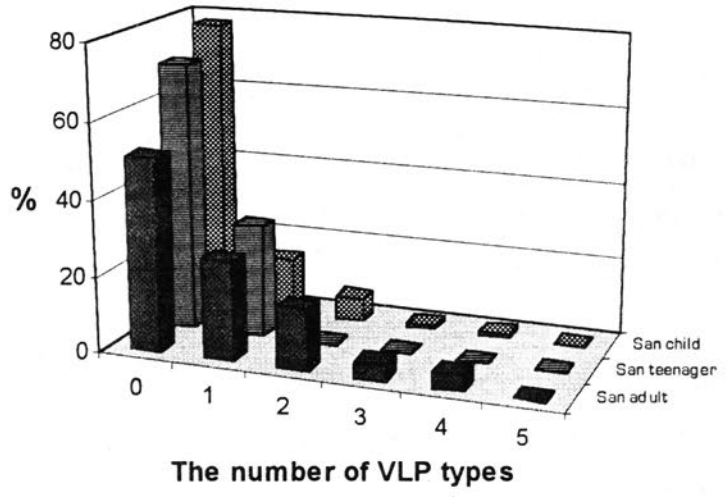

Fig. 4.1. The percentage of seronegative individuals amongst the San people and the percentage of those seropositive to 1, 2, 3, 4, or 5 VLP types.

In an attempt to ascertain possible serological cross-reactivity between the different VLP types, scatterplot analysis of the corrected OD values obtained for each serum against each VLP types was compared to all of the other VLP types.

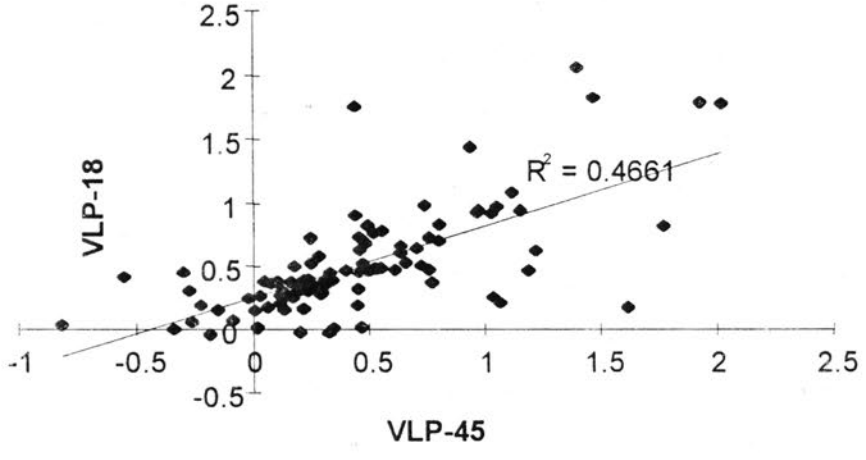

Fig. 4.2. A comparison by scatterplot analysis, of the seroreactivity of San women to VLP-18 and VLP-45.

A weak correlation was found, between VLP-18 and VLP-45 (Fig 4.2) in the San adult group ($R^2 = 0.466$). For all the other VLP types, R^2 value was < 0.1 when compared with one another.

4.4. DISCUSSION

The first trial of a candidate HPV VLP vaccine in humans is underway [IARC/WHO, 1999]. This raises the hope of the availability of HPV vaccines in the near future. Indications are that the immunity induced to HPV is type-specific [Rose *et al.*, 1994b; Roden *et al.*, 1996] and vaccination with one type may be unlikely to protect against infection with another type. A study of the HPV types in the Southern African population is necessary to determine the exposure to the major oncogenic types that exist, if HPV vaccines are to be introduced. This study examined the differences in seropositivity to 5 oncogenic HPV VLP types amongst the San people.

San children had low antibody seropositivity to all of the VLP types and this compares with results described in Cape Town children for VLP-16 in Chapter 2.3.2. and for VLP-16, -18, 31, -33 and –45 in Chapter 3.3.1.2. However if the results of the tests to individual VLP were combined then 22.6% of the San children were seropositive to at least one HPV type. This result correlates with the assessment of the seroreactivity of 115 children in Cape Town (Chapter 3.3.3.) to multiple VLP types, where seropositivity to at least 1 type was 27.3%. More of the children < 8 years were seropositive (13/46, 28.2%) than those > seven years (13/69, 18.8%) suggesting that the seroprevalence rate is less with an increase in age and that infection occurs early in life. The reason for the high incidence of antibodies in male San children (18/54) compared with female children (8/61) is unclear as no such difference was found in the Cape Town children (Chapter 3.3.3.). A difference in the mode of HPV transmission is implicated between the children of different sexes, because of the higher rate of antibodies in the male children. However in male adults the reverse is true, with a higher percentage of adult women having anti-HPV antibodies [Strickler *et al.*, 1999]. Also no gender difference in seroprevalence was noted for the Cape Town children (Chapter 3.3.3.), so possibly the gender difference in the San children was co-incidental.

Almost half of the San women and more than a third of the children, who were HPV VLP seroreactive were positive to more than one VLP type. The relevance of the simultaneous detection of antibodies to more than one type of VLP can be explained in terms of multiple infections or serological cross-reactivity as described in Chapter 3.4. There is also a greater combined seroreactivity in San women (14/25), to both VLP-18 and VLP-45 than other types, which correlates with their phylogenetic relatedness as was noted in Chapter 3.3.3.5.

The highest seroprevalence rates in the San adults were to VLP-18, -33, and –45. High seroprevalence of antibodies to VLP-18 and VLP-33 amongst normal healthy control women has been described [Chua *et al.*, 1996], which did not confer an increased risk of cervical disease, whereas seroprevalence to VLP-16 conferred a 3-fold increased risk of developing CIN. In a study comparing VLP-16 seroprevalence in blood donors in the United States (US) and Jamaica [Strickler *et al.*, 1999], where there is a 3 fold higher incidence of CaCx, age adjusted seoprevalence rates for VLP-16 were 12% and 24% respectively. Blood donors in Cape Town have a VLP-16 seroprevalence of 25.3% (Chapter 3.3.1.2.). The San VLP-16 antibody seroprevalence was 16.8%, suggesting a lower exposure to HPV-16 and consequently a lower risk of CaCx. Previous studies [Bloch *et al.*, 1988] on the prevalence of genital tract HPV in the Namibian region, showed a very high incidence of both CIN and genital HPV types in the people of that area. The San group in this investigation included people from the same regions of Namibia as in that study. The San women had seroprevalence levels for VLP-18, -33 and -45, that were higher than those reported for blood donors in Cape Town (VLP-18 10.5%, VLP-33 8.4% and VLP-45 13.7%) (Chapter 3). This indicated a higher exposure to these HPV types in the San community compared with the Cape Town blood donors. In general, the San people have only one sexual partner, once married. Although the younger San people have more than one sexual relationship, a high degree of promiscuity is not common. Only one case of CaCx has been reported in the last 5 years in the community of 4500 people [Captain Pretorius, personal communication]. However, since seropositivity to oncogenic HPV types was evident in 50% of San adults, cervical infection in the women, or infection at sites other than the cervix cannot be ruled out.

A limited amount of data is available on the HPV types present in South African communities. In Cape Town, HPV-16 has been reported to be the predominant type in women with CIN and CaCx [Williamson *et al.*, 1989; Williamson *et al.*, 1994], while in women with normal cytology, the predominant type is HPV-18 [Ramesar *et al.*, 1996]. *In situ* hybridisation studies to establish which HPV types were associated with CIN and CaCx in Durban indicated that HPV-16 was the predominant type but the "minor types" of HPV, ie. other than HPV-16, might be more common in this region compared with elsewhere [Cooper *et al.*, 1991a; Cooper *et al.*, 1991b]. Some information is available on HPV types present in central and northern African countries. Bosch *et al.* [1995] indicated a significant increase in HPV-45 DNA amongst CaCx patients in African countries in their

study, compared to other areas of the world. HPV-18 DNA in Tanzania [ter Meulen *et al.*, 1992] and Uganda [Schmauz *et al.*, 1989], has been found to be more prevalent amongst CaCx and non-cancer patients than other areas of the world. In Senegal, a high proportion of patients with cervical lesions were shown to be infected with HPV-18 (39%) and HPV-45 (10%) and HPV-18 DNA was found in 7% of pregnant women [Chabaud *et al.*, 1996].

This study has contributed information on the presence of antibodies to five HPV VLP types in San people. These findings regarding the San people corroborate other evidence both amongst CaCx and non-cancer individuals that HPV-18 and -45 are more prevalent in certain parts of Africa than elsewhere. It remains be established whether San women with antibodies to these VLP types are at risk of disease.

CHAPTER 5. A STUDY OF CERVICAL AND ORAL MUCOSAL IMMUNITY IN WOMEN WITH CERVICAL DISEASE

Women with cervical disease display anti-VLP-16 IgA antibodies in oral fluids and HPV-16 positive women with CIN anti-VLP-16 cervical and serum IgG antibodies

5.1. INTRODUCTION

It is accepted that genital HPV infections are acquired sexually [Dillner *et al.*, 1999] and involve contact of the virus with the genital tract mucosal epithelium. The presence of local antibody at mucosal surfaces correlates with the protection against viral pathogens in humans and experimental animals [Murphy, 1999]. Therefore a study of the local immune response at the cervical mucosa in women with cervical disease should contribute to our understanding of the immune response to HPV. Mucosal surfaces have also been shown to be part of the common mucosal immune system (CMIS) as discussed in Chapter 1.11.4.2. It would be expected therefore that infection at the genital mucosa should produce an antibody response at the oral mucosa.

Salivary HPV antibodies could be indicative of cervical disease as part of the CMIS. The screening of oral samples for HPV antibodies has not been reported, but this method may provide a convenient, alternative non-invasive method to serology or HPV DNA assessment for the identification HPV presence or markers of cervical disease. Oral immunisation is known to activate the common mucosal-associated lymphoid tissue (MALT) and induce antibodies not only in oral but also in cervical secretions of both mice [Balmelli *et al.*, 1998] and humans [Rudin *et al.*, 1998]. The oral route has been investigated for the administration of potential HPV VLP vaccines [Rose *et al.*, 1999]. Conversely, immune stimulation of the vaginal mucosa with cholera toxin B (CTB) subunit has been shown to produce anti-CTB IgA antibodies in salivary secretions [Kozlowski *et al.*, 1997]. Equally, immune stimulation at the genital mucosa by HPV could elicit HPV-specific antibodies in oral secretions although there is no experimental evidence of this.

The testing of oral samples has a considerable number of advantages over venipuncture or the procurement of biopsy material for the determination of HPV infection [Malamud and Fiedman, 1993]. The technique is non-invasive, which would promote patient acceptance of and compliance with testing as well as being accepted for use with children and adolescents. Oral testing would not require a heath-care worker with any specialised training and is ideally suited to field collection in a developing country such as South Africa, where a large part of the community live in rural areas, far from hospitals or clinics. The risk of needle-stick injuries would be removed as well as other the risks from blood contamination. Studies have indicated that certain proteases in human oral fluid render HIV non-infectious [Robinovitch *et al.*, 1993], making these samples less biohazardous than blood. The test would also have application in the vaccine trials, both for initial screening and subsequent monitoring of immune responses to potential vaccines.

Oral testing has been used successfully to detect HIV antibodies, where anti-HIV antibodies were detected in oral fluids in all but one of 36 seropositive individuals [Holstrom *et al.*, 1990] and 142 of 145 seropositive individuals [Behets *et al.*, 1991]. All seronegative patients were saliva negative. There is evidence to suggest that HIV immune conversion occurs simultaneously in serum and oral fluid [Connell *et al.*, 1993]. More recent assays have confirmed the sensitivity of the HIV antibody oral test [Gallo *et al.*, 1997; Granade *et al.*, 1998]. Hepatitis C virus antibodies have been similarly detected by ELISA in oral fluid with a 90% sensitivity [Elsana *et al.*, 1998] and antibodies to *Trypanosoma cruzi* with a sensitivity of 90.4% in infected patients [Pinho *et al.*, 1999].

Oral fluid consists of secretions of salivary glands (saliva), gingival crevicular fluid (GCF), bacteria and particulate matter [Emmons, 1997]. GCF is the fluid that accumulates round the necks of the teeth, between the teeth and the gum. It is a transudate from serum, formed from the continuous seepage of fluid from gingival capillaries through the crevicular epithelium and into the gingival crevice between the teeth and gum. Saliva is the main component of oral fluid, and secretory IgA (SIgA) is its main immunoglobulin, mostly appearing as dimeric, but 5-10% as monomeric IgA [Challcombe and Shirlaw, 1999]. The IgA1 to IgA2 ratio is about 55:45. In whole saliva, the concentrations of SIgA, IgG and IgM are approximately 200, 2 and 1 mg/ml, respectively [Challcombe and Shirlaw, 1999]. The composition of GCF more approximates that of serum, but once

mixed with saliva, its immunoglobulin concentrations fall significantly [Challcombe and Shirlaw, 1999]. The levels of IgG in whole oral fluid are 300 times less than in GCF, which is 4 times less than in serum and the levels of IgA in saliva 6 times less than in GCF which is 2 times less than in serum.

This study aimed to assess the advantages of the oral screening of antibodies to HPV, to compare this with serum antibody detection, and to investigate the association of these antibodies with CIN. The study also aimed to investigate the association of CIN with antibodies at the cervical mucosa. The interpretation of serum antibody responses to HPV is hampered by the fact that genital HPV types can be recovered from a number of non-genital sites [Shah and Howley, 1996], including the oesophagus, conjunctiva, sublingual region and the buccal mucosa. A serological response to HPV may therefore not necessarily indicate genital exposure and a study of a local cervical response may be more successful at detecting genital HPV. Specific immunoglobulin identified locally in cervical secretions could eliminate the possibility of cross-reactivity with cutaneous HPV or with other HPV from other sites. The identification of cervical IgA as responsible for the HPV-specific reactivity could confirm the mucosa as the site of the specific response and so an assay of local secretions could uniquely predict a viral association with cervical disease [Roche and Crum, 1991]. Although there could be HPV-specific IgA responses at mucosal sites other than the cervix by way of the common mucosal immune system (CMIS) [Mestecky, 1987], the major IgA response would probably be at the site of the initial stimulation, the cervix [Hanneberg *et al.*, 1994; Kowlowski *et al.*, 1997]. A study of the local immune status of women with cervical disease may give crucial insights into the understanding of the immune phenomena that occur during the development of early disease and possible immune factors which contribute to progression to CaCx.

It has been shown that 50%-70% of persons with HPV-associated neoplasia have serum antibody responses to HPV antigens [Kirnbauer *et al.*, 1994; Wideroff *et al.*, 1996; Nonnenmacher *et al.*, 1999]. The local cervical immune response was first tested by Dillner *et al.* [1989] using purified BPV virions in ELISA and found a correlation between anti-BPV IgA in cervical mucus and serum IgA in women with CIN, but not for anti-BPV IgG. There was an increase in the proportion of positive anti-BPV IgA in cervical secretions of women with CIN compared with normal healthy women. This was surprising

as antibodies to intact PVs are considered type specific so this study probably detected cross-reactive antibodies to disrupted virions (Heino *et al.*, 1995). In the investigation of HPV DNA and anti-HPV IgA antibodies in cervical secretions of normal women using synthetic HPV-16-specific peptides as antigen, Veress *et al.* [1994] found a low correlation between HPV DNA and anti-HPV IgA positivity. That study suggested that this was possibly due to the fluctuating course of latent HPV infection and presumed fluctuating IgA levels in non-persistent infection. Veress *et al.* [1994] showed a relationship between IgA positivity and age, with IgA prevalence being highest in women aged 25-32 years, and significantly lower in the younger and older groups. Wang *et al.* [1996] studied the relationship between cervical mucus antibodies to VLP-16, -18 and –33 and the presence of viral DNA in women with cervical disease. A HPV VLP type-restricted IgA response was detected against these 3 VLP types which was associated with a concomitant HPV infection. More recently, Bontkes *et al.* [1999] investigating the role of HPV-16 VLP-specific antibodies with respect to clearance of HPV-16 and premalignant disease in women with abnormal cytology, found that a systemic not a local IgA response was related to viral clearance. Virus-specific antibodies induced in the mucosa may not be effective in eliciting the regression of established lesions but HPV-specific local immune responses may however be important in the early stage of infection, blocking re-infection and preventing viral persistence by viral clearance.

Knowledge of the type of immune response at mucous membranes is essential therefore, not only to enhance protection against HPV and decrease infections, but also it is a prerequisite to the design of anti-HPV vaccines. This Chapter will describe the first reported successful detection of anti-HPV VLP-16 IgA antibodies in oral fluids and will demonstrate the significant association of these antibodies with CIN. Oral antibodies will be compared with those in serum and related to the HPV DNA status of women with CIN and the significance of oral fluid HPV antibody testing as markers of disease will be discussed. The success of the HPV antibody detection in the oral fluids, prompted an expansion of the study to include the determination of the HPV-16 DNA status in buccal cells to establish whether the mouth was the site of HPV infection. Also determined was the HPV-16 VLP antibody prevalence in the cervical mucus of women with CIN to study the nature of the local immune response at the site of infection, the cervix, and to compare this with the antibody responses in serum and oral fluids. It was hoped to gain an insight into the humoral responses at systemic and mucosal sites in women with cervical disease

and relate this to HPV infection as demonstrated by DNA analysis. A study of this nature, correlating the assay results of samples from such a large number of sites within the same person, has not previously been reported.

5.2. MATERIALS AND METHODS

5.2.1. Study populations

The study participants were 88 women with CIN or CaCx attending colposcopy clinic or receiving in-patients care at Groote Schuur Hospital, Cape Town during 1997. Approval for this study was obtained from the Ethics and Research committee of the University of Cape Town. Women were informed as to the nature of the study and gave signed consent to the removal of the various specimens. All specimens were collected at the same time, but not all samples were obtained from all the patients. By histology there were 15 with CIN 2, 67 with CIN 3 (i.e. 82 with CIN2/3) and 6 with CaCx (squamous). The average age of the 88 patients assessed was 38 years (range 19-76 years). Sera were obtained for the estimation of serum ELISA cut-off values, from 28 children (aged 3-12 years) attending Cape Town hospitals for undefined reasons. This group of children was different to that described in Chapter 2 and 3, as there were not sufficient serum samples remaining from the initial group of children.

5.2.2. Sample collection and preparation

5.2.2.1. Oral samples and serum

Oral fluid samples and serum were obtained from 73 women with CIN2/3 and 6 with CaCx (squamous). Oral fluid was collected by the insertion into the mouth of a commercial absorbent OraSure pad (Epitope, Beaverton, Oregon, USA), attached to a plastic stick, specifically designed for the collection of oral mucosal transudate also known as gingival crevicular fluid (GCF). The pad was first rubbed back and forward several times and then

kept stationary for two minutes in the gingival area of the lower mouth. The pad was removed and placed in a collection tube with OraSure buffer (Epitope, Beaverton, Oregon, USA) and stored at –20^{0}C until required. Control oral samples were collected from 36 healthy laboratory volunteers, who consisted of 21 females (12 younger than 36 years, 9 older than 35 years) and 15 males (8 younger than 36 years and 7 older than 35 years).

5.2.2.2. Cervical mucus and cervical cells

Cervical mucus (CM) samples were obtained from 26 women with CIN (4 with CIN2 and 22 with CIN3). Collection entailed removal of the mucus from the endo-ecto cervix with a small cotton swab attached to a narrow, wooden stick, which was then inserted into a plastic tube and sealed and stored at –20 ^{0}C. The cervical mucus samples were prepared for ELISA by placing the cotton swab containing the mucus with a small portion of the attached shaft in a 2ml Eppendorf tube with 300µl of PBS containing 4mM of the protease inhibitor, Pefabloc (Boehringer Mannheim, Germany) and 5% milk powder (Elite). The volume of mucus did not vary considerably and was approximately 100µl. The tubes were vortexed vigorously for 1-2 min and then centrifuged in a microfuge at 14000rpm for 5 min and the supernatant fluid used immediately for ELISA Ig, or stored at –20 ^{0}C. Cervical punch biopsy material or cervical cells, adequate for HPV DNA determinations, were obtained from 45 women. Cervical cells were collected by gently swabbing of the ectocervix with a brush (Cervi-brush, Pharmaceutical Enterprises, Cape Town) and the brush placed in a tube and stored at –20 ^{0}C.

5.2.2.3. Buccal cells.

Buccal cells were collected from 28 women on a Cervi-brush by twirling the brush in the mouth, against the cheek. The brush was placed in a tube, sealed and stored at –20 ^{0}C.

5.2.3. ELISA

The VLP-16 preparation for ELISA was kindly supplied by MedImmune (USA), via Dr. Robert Rose, Rochester University and the BPV-VLP preparation supplied by Dr. Rose.

The ELISAs were conducted as described in Chapter 2.2.3, with some alterations in protocol. The VLPs were used at a coating concentration of 0.2μg per well or 2μg /ml (not 10μg/well, as for previous VLP ELISAs). The oral samples were diluted 1:1 in M-PBS and the cervical mucus (CM) samples 1:5 in M-PBS. The CM sample OD values and the cut-off values were estimated for IgA and IgG using the HPV DNA negative groups mean ELISA OD value plus 2SD. The difference between negative and positive values was estimated as being at cut points of (A_{492}) 0.2 for the IgG and 0.1 for the IgA ELISA values. These were confirmed by viewing scatterplot analysis of the absorbance values obtained for the test groups for the IgA and IgG ELISAs (not shown). A cut-off for the oral fluid OD values was estimated using the control group oral fluid mean OD value plus 2SD as described before in Chapter 2.2.4. The cut-off value for positive oral fluid estimation was set at an absorbance of (A_{492}) 0.47. The total IgA and IgG contents of OF and CM were determined by ELISA as described by Rudin *et al.* (1998). Briefly, the plates were coated overnight at 4^0C with goat anti-human IgA, alpha chain specific or IgG, specific for gamma-chains (Dako, Carpinteria, CA, USA). After washing plates as for previous ELISAs, samples and standards (Dako, polyclonal human IgA or IgG) were added serially diluted. Bound total IgA or IgG antibodies were determined as described in Chapter 2.2.3. The concentrations of total IgA or IgG in samples was assessed using the standards.

5.2.4. PCR

All PCR procedures on cervical or buccal cell material were performed by Patti Kay and Bruce Allen. A 200μl aliquot of thawed sample was removed for DNA extraction by the method of Qiagen (Qiagen, Hilden, Germany). The quality of the DNA was tested by amplification of the CCR5 gene [Michael *et al.*, 1997]. An HPV-16 specific primer [van den Brule *et al.*, 1989] was used for HPV-16 DNA L1 amplification on cervical cells. A two stage PCR was conducted on buccal cells using nested degenerate primers [Williamson and Rybicki, 1991] to amplify L1 regions of HPV DNA. Buccal cells were HPV-16 typed by restriction fragment length polymorphism using the restriction enzyme BstE II (Boehringer Mannheim) [unpublished data].

5.3. RESULTS

5.3.1. Anti-VLP-16 IgA in oral fluids of women with cervical disease and normal controls

To investigate whether the prevalence of antibodies in oral fluids could serve as a marker of cervical disease, the oral fluids of 79 women (73 with CIN2/3 and 6 with CaCx) and 36 normal controls were tested by ELISA for the presence of anti-VLP-16 antibodies. The major form of IgA in salivary and cervical fluids is secretory IgA (SIgA) [Challcombe and Shirlaw, 1999; Russell *et al.*, 1999]. In referring to both fluids the general term of IgA will be used to describe SIgA and the minor IgA constituent, monomeric IgA (mIgA), which is not secretory. IgA was detected in the oral fluid of the majority of women with cervical disease, but the assay was not sufficiently sensitive to detect IgG antibodies. The boxplots of the corrected OD values obtained in the oral fluid IgA ELISAs are shown in Fig. 5.1

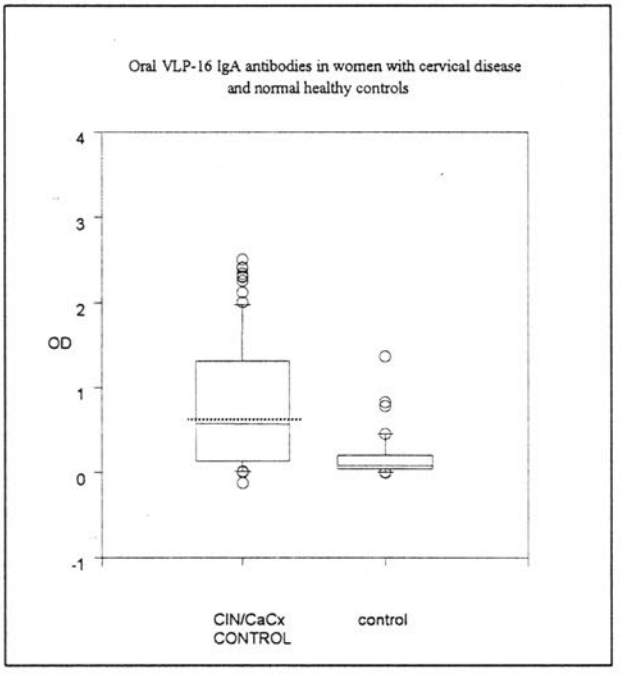

Fig. 5.1. Boxplots of the OD values obtained in VLP-16 ELISA to detect oral IgA antibodies in 79 women with cervical disease (CIN/CaCx) and 36 normal healthy controls (controls). The boundaries of the box represent the 25 and 75 percentiles and the line in the box the median value. Error bars represent the 10^{th} and 90^{th} percentiles. The cut-off value for positivity was OD 0.47 (dotted line).

The prevalence of anti-VLP-16 IgA in oral fluids of women with cervical disease was 55.7% (44/79), significantly higher than the 8% (3/36) prevalence rate found in the control group (P =0.000002).

5.3.2. Serum and oral antibody prevalence rates in women with cervical disease and oral IgA in healthy controls

In order to determine how the immune response at the oral mucosa related to the systemic response, oral IgA in the women with cervical disease were compared with their serum antibodies. Women with cervical disease were assessed for their anti-VLP-16 IgA and IgG antibody responses in serum, and the serum prevalence rates compared with oral IgA antibody prevalence rates (Table 5.1). The oral IgA response of normal controls was also tabled.

Table 5.1. The prevalence of anti-VLP-16 serum IgA and IgG in women with CIN2/3 and CaCx and oral IgA in women with CIN2/3, CaCx and controls

Test	CIN2/3 n=73		CaCx n=6		Controls n=36	
	No positive	%	No positive	%	No positive	%
Oral IgA	41	56.2	3	50.0	3	8.0
Serum IgA	34	46.6	3	50.0	nd	
Serum IgG	41	56.2	3	50.0	nd	

nd = not done

There was a 56.2% prevalence of anti-VLP-16 oral IgA antibodies in women with CIN2/3, (which was increased to 60% in the 15 women with CIN 2). More women with CIN2/3 had oral IgA antibodies compared with serum IgA (46.6%), with 19 of the 34 (55.9%) serum IgA positive women having a corresponding oral IgA antibody presence. The same number of CIN2/3 patients had oral IgA as serum IgG (56.2%), with 22 of the 41 (53.7%) serum IgG positive women having corresponding oral IgA antibodies. Fifty percent of the CaCx patients had oral IgA and serum IgA and IgG antibodies, but the CaCx patient group was too small for any comparative analysis.

5.3.3. Serum and oral antibodies and HPV-16 DNA status

The HPV DNA status was determined on 45 of the 79 patients with cervical disease (4 with CaCx and 41 with CIN2/3) who had been tested for anti-VLP-16 serum and oral antibodies. Of these 45 patients, 19 were HPV-16 positive (42.2%). A comparison was made between the responses of the 19 HPV-16 positive women and the 26 patients that were not HPV-16 positive (non-HPV-16) (Table 5.2.) to determine the type specificity of the relevant assays.

Table 5.2. A comparison of the oral and systemic antibody responses in HPV-16 positive women and those not HPV-16 positive (non-HPV-16).

Test	HPV-16 positive		Non-HPV-16		*P* value
	n/total	%	n/total	%	
Oral IgA	11/19	57.9	16/26	61.5	0.80
Serum IgA	8/19	36.8	13/26	50.0	0.60
Serum IgG	12/19	63.2	11/26	42.3	0.16

There was no significant difference between HPV-16 positive and non-HPV-16 women with regard to oral anti-VLP-16 IgA or systemic anti-VLP-16 IgA and IgG. The largest difference was between the serum IgG responses of the two groups, which appeared the most accurate indicator of HPV-16 infection. More HPV-16 positive women showed an anti-VLP-16 serum IgG response (63.2%) compared with serum IgA (36.8%) or oral IgA (57.9%) responses. More women who were not HPV-16 positive responded with IgA antibodies both systemically and orally than those who were HPV-16 positive.

5.3.4. The nature of the local cervical immune response in women with CIN compared with that in serum and oral fluid and the relationship of the responses with cervical and buccal HPV-16 DNA status

In order to determine the nature of the local immune response (at the cervix) in women with CIN, anti-VLP-16 antibody prevalence in cervical mucus (CM) was determined in a group of 26 of the previously described women with CIN2/3 (4 with CIN2 and 22 with CIN3). This response was compared with their antibody prevalence rates in serum and oral fluid and was also related to their HPV-16 DNA status at the cervix and in the mouth.

5.3.4.1. Cervical, serum and oral antibodies in women with CIN

Anti-VLP-16 cervical mucus IgA and IgG antibodies were detected by ELISA in 26 CIN patients and the cervical mucus antibody OD values obtained were compared with the OD values obtained in serum and oral fluid antibody ELISAs by boxplot analysis (Fig. 5.2.).

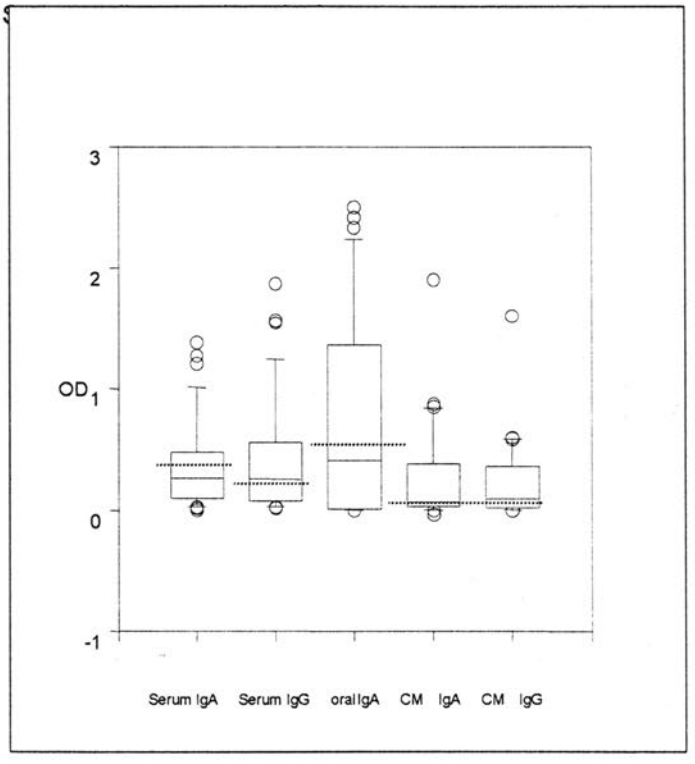

Fig. 5.2. Boxplot analysis of OD values obtained in IgG and IgA VLP-16 ELISA with serum, oral fluid and cervical mucus of women with CIN2/3. The boundaries of the box represent the 25 and 75 percentiles and the lines in the boxes the median value. Error bars represent the 10^{th} and 90^{th} percentiles. Dotted lines represent the cut-off values for ELISA positivity.

The boxplots could not be directly compared because of the different dilution factors for the different fluids (serum 1:20, oral fluid 1:1 and CM 1:5). The percentage of women with CIN positive for antibodies to VLP-16 was serum IgA 65.4%, serum IgG 61.5%, oral IgA 56.5%, CV IgA 42.3% and CV IgG 38.5% (Table 5.2.).

5.3.4.2. A comparison of antibody responses and HPV-16 DNA status.

The HPV-16 DNA status of the women with CIN2/3 was compared with the anti-VLP-16 antibody responses systemically, locally at the cervix and in the oral fluid of these patients and is presented in table 5.3.

Table 5.3. Serum, oral and cervical anti-VLP-16 antibody responses in CIN2/3 patients and their HPV-16 DNA status at the cervix and mouth.

Patient		Serum		Oral fluid	Cervical mucus		DNA Status	
Age	CIN	IgA	IgG	IgA	IgA	IgG	Cervical HPV16	Buccal HPV16
25	2	+	+	ns	+	+	+	-
27	2	-	-	-	+	-	ccr5-	+
31	2	+	+	ns	-	+	-	-
27	2	+	+	+	-	-	-	-
39	3	-	+	+	-	+	-	-
39	3	+	-	+	+	-	-	
36	3	-	-	+	-	-	+	
28	3	-	+	+	-	+	+	-
40	3	-	-	-	+	-	-	-
42	3	+	-	+	-	-	ccr5-	-
45	3	+	+	-	+	+	+	
33	3	-	+	-	+	-	-	+
52	3	+	-	+	-	-	ccr5-	
38	3	+	+	+	+	+	+	-
29	3	-	+	-	-	+	-	
39	3	+	+	-	-	-	-	-
41	3	-	+	-	+	-	ccr5-	-
42	3	+	+	+	-	-	-	
43	3	+	+	+	+	+	-	-
32	3	+	+	+	-	-	+	
45	3	+	+	-	-	+	+	-
38	3	+	-	+	-	-	-	-
29	3	+	-	-	+	-	-	ns
45	3	-	+	ns	-	+	+	ns
25	3	+	-	-	+	-	-	
39	3	+	-	+	-	-	-	-
Total positive	/total tested	17/26	16/26	13/23	11/26	10/26	8/22	2/24
Percent positive		65.4	61.5	56.5	42.3	38.5	36.4	8.3

+ = positive, - = negative, ns = no sample available, ccr5- = no amplifiable DNA.

Marginally more women demonstrated serum IgA (65.4%) responses rather than serum IgG (61.5%) responses. Both serum IgA and IgG anti-VLP-16 antibodies were present in more

CIN patients than the oral IgA (54.2%) antibodies. There was a 36.4% prevalence of HPV-16 DNA at the cervix of the CIN patients compared with a 8.3% HPV-16 DNA prevalence in buccal cells. There was no correlation between the HPV-16 DNA prevalence in buccal cells and oral IgA, indicating that the site of infection was possibly at the cervix. The number of individuals who displayed a corresponding antibody response both systemically and locally, or systemically and orally, or locally and orally are shown in Table 5.4.

Table 5.4. Correlates of VLP-16 antibody positivity for the same women in cervical, oral and serum samples.

Comparison of	with	Number correlating	%
Serum IgG	Cervical IgG	10/10	100
Serum IgA	Cervical IgA	7/11	64
Oral IgA	Cervical IgA	3/11	27
Serum IgA	Oral IgA	10/13	77

There was a 100% correlation between those women with an IgG response at the cervix and a serum IgG response, with a 64% correlation for the corresponding serum versus cervical IgA responses. There were 77% of women positive for serum IgA who were correspondingly positive for oral IgA antibodies. Of those with cervical IgA, 27% had oral IgA antibodies. Despite the diversity of the antibody responses, three women displayed IgA and IgG antibodies in all three fluids and a further three had antibodies of at least one type in all three fluids. Four women had antibodies in only one of the fluids tested and there were no patients who did not show an Ig response in at least one fluid sample.

The antibody responses systemically, locally at the cervix, and orally were compared with regard to their cervical HPV-16 DNA status and the results are tabled (Table 5.5)

Table 5.5. The comparison between salivary, systemic and local, cervical (CM) responses according to HPV DNA status

Test group	oral IgA		Systemic response				Local (CM) response			
	IgA		IgA		IgG		IgA		IgG	
	n	%	n	%	n	%	n	%	n	%
HPV–16+	4/8	50	5/8	62.5	7/8	87.5	3/8	37.5	6/8	75
Non-HPV-16	6/14	42.9	10/14	71.4	8/14	57.2	6/14	42.9	5/14	35.7

There were no statistically significant differences between the number of VLP-16 IgA or IgG responders that were HPV-16 DNA positive (HPV-16+) women compared with women not HPV-16 positive. The largest differences were between HPV-16+ (7/8) and non-HPV-16 (8/14) women for serum IgG (P= 0.16) and HPV-16+ and non-HPV-16 women for cervical IgG (6/8 and 5/14, P = 0.09). These results indicate that IgA responses were similar regardless of HPV status but HPV-16 DNA positive women were more likely to have IgG responses systemically and locally than women not HPV-16 positive.

Systemic responses correlated well with the presence of cervical HPV-16 infection as well as local (CM) IgG responses, but CM anti-VLP-16 IgA antibodies did not correlate well with HPV-16 positivity (37.5%) and there was a 50% oral anti-VLP-16 IgA correlation with HPV-16 DNA positivity at the cervix.

5.4. DISCUSSION

This chapter investigated the VLP-16 antibody responses of women with CIN both at the oral and cervical mucosal surfaces and in serum. The detection of anti-VLP-16 oral IgA in women with CIN was the first report of HPV antibodies in oral fluid. The detection of anti-VLP-16 IgA antibodies and their significant association with cervical disease has application in the screening of women at risk of cervical disease by oral testing. Oral IgA anti-VLP-16 responses were found to correlate well with the serum IgA responses in the same woman. Serum IgA and IgG responses (in 65.4% and 61.5% of women respectively), were the antibody responses detected in most CIN patients. Cervical IgA and IgG responses were found less frequently (42.3% and 38.5%, respectively). All the women who had cervical IgG had corresponding serum IgG antibodies and it was the anti-VLP-16 IgG responses both systemically and locally at the cervix which were found most prevalent in women HPV-16 DNA positive at the cervix.

The OraSure device used for oral fluid collection is designed to collect oral serum transudate in crevicular fluid [Gallo et al., 1997]. It preferentially does so as the collection pad creates an osmotic gradient to stimulate the transudation of interstitial fluid and to

concentrate it onto the pad which should contain predominately GCF [Gallo *et al.*, 1997]. The sample collected by this proceedure should have IgG levels 4-fold greater than those found in whole saliva. The GCF volume is between 1-2 ml per day and mixes with saliva from salivary glands at a dilution of 1:500 and 1:1000 [Challacombe and Shirlaw, 1999], but it is unclear to what degree the GCF and saliva were mixed in the samples of the present study. The IgG levels in the oral fluid collected in this study were too low for detection by VLP ELISA, although the total IgG levels were within normal ranges, and the IgA levels very high. It was assumed therefore that there was a large amount of saliva in the oral samples as saliva has a higher concentration of IgA than IgG [Malamud, 1997] and would dilute the amount of GCF IgG but increase the overall IgA concentration. The excess amount of IgA in the oral samples was probably due to the collection procedure of the oral fluid, which collected predominately saliva. However the detection of salivary IgA in the oral fluid was of interest in the context of the CMIS. It represents the first indication that HPV infection at the cervical mucosa induces HPV-specific secretory IgA antibodies at the oral mucosa.

The large number of CIN patients with oral anti-VLP-16 IgA suggested that the IgA antibodies were either locally produced or as a result of stimulation through the CMIS. Although local stimulation of the oral mucosal immune system via oral sex must be considered, it is improbable as anti-VLP-16 oral IgA was found in most women and should have been associated with a greater prevalence of HPV-16 DNA in the mouth, which was not found. The high correlation between systemic and salivary IgA was unexpected as it has been calculated that only small amounts of salivary IgA are derived from the circulation and the remainder is synthesised locally in salivary glands [Mestecky, 1987]. Systemic IgA produced by the bone marrow B cells, the primary source of serum IgA and secretory IgA (SIgA) produced by B cells mucosally, differ significantly in molecular properties [Kutteh *et al.*, 1999]. Serum IgA is predominately monomeric and salivary SIgA is predominately dimeric. It is therefore unlikely that the oral IgA detected in this study was derived from serum, despite the fact that a good correlation was found between serum IgA and oral IgA in the same person. It is unclear however how systemic and mucosal immune systems interact with one another [Balmelli *et al.*, 1998] and serum IgA levels might influence the levels of salivary IgA. The most likely origin of the salivary IgA, however, was via the CMIS, with the inductive site possibly at the site of infection, the cervix.

The detection rate of salivary anti-VLP-16 IgA in women with CIN 3 was 53%. An increased prevalence rate of 60% in women with CIN 2 could make the test applicable for use in women with early stage CIN disease. Anti-VLP-16 specific IgA antibodies were similarly detected in women with HPV-16 DNA and non-HPV-16 positive women. However the antibodies in the latter group could reflect past infection that has been cleared in these women or infection at another site. There was no correlation between anti-VLP-16 salivary IgA and buccal cell HPV-16 presence, indicating the infection was possibly at the cervix. However, the correlation of salivary VLP-16 IgA and cervical HPV-16 DNA was only 20% and as the cervical mucosa comprises part of the CMIS similar antibody expression would be expected throughout the CMIS., Compartmentalisation within the CMIS does however occur [Moldoveanu *et al.*, 1995] and cervical HPV stimulation might not directly induce cervical antibodies to the same efficiency as oral antibodies. It will be important to assess the degree to which stimulation of the cervical mucosal system by HPV-16 (or VLP-16) in women causes HPV antibodies in saliva before conclusions about a common mucosal response can be made.

Cervical antibodies have been shown to decrease more rapidly with time compared with those in serum [Friedman *et al.*, 1989], and might also decrease faster than those in saliva. Cervical antibodies also have been shown to be under hormonal control and vary with the menstrual cycle [Kutteh *et al.*, 1998]. The stage of CIN patients' menstrual cycle was not known when the CM samples were obtained and this should be taken into account in further studies. Oral, rectal and vaginal immunisation of women with cholera toxin B subunit (CTB) all produce a significant increase in specific salivary IgA, reaching a peak 2 weeks after immunisation [Kozlowski *et al.*, 1997]. Anti-CTB IgG antibodies were not detected in salivary secretions from any of the immunisation routes, presumably because of low levels of IgG in saliva. The present study was also not able to detect anti-VLP-16 IgG in oral fluids and it was assumed that there were low levels of IgG antibodies that could not be detected by the assay or that the high levels of IgA blocked the binding of the IgG to the VLPs.

The analysis of the nature of anti-VLP-16 antibody responses at different sites emphasised the complexity of the humoral response to HPV infection. All of the women however, showed an immune response of at least one isotype at at least one site and 23% (6/26) of

the women showed antibody responses at all sites investigated. The anti-VLP-16 IgG serum antibody response, occurred in 7 of the 8 HPV-16 DNA positive women, compared with 57.1% (8/14) in non-HPV-16 women (P = 0.16). Likewise the anti VLP-16 CM IgG responses were also type-specific, occurring in 75% (6/8) of HPV-16 positive CIN patients but only in 35.7% (5/14) of non-HPV-16 women (P = 0.09). The IgA responses in CM and serum occurred in more non-HPV-16 women than those HPV-16 DNA positive and in oral fluids to almost the same degree in non-HPV-16 positive women as HPV-16 positive women and could reflect a cleared infection. Women all differed in their type of response (systemic, local, and oral) and in the levels of antibodies found at the different sites, as well as relative to their HPV DNA status. This may be due to serum antibodies to VLP-16 (and possibly elsewhere) reflecting a cumulative lifetime exposure to HPV-16 [Carter *et al.*, 1996] and not only to the HPV infection under study. The number of women involved in the study was too small to distinguish significant trends in the relevant responses. Longitudinal studies, examining the different antibody responses over time and comparing these with HPV infection acquired and the subsequent development of disease, will be the only method of more clearly defining the antibody responses to HPV infection.

Serum and CM antibody responses correlated well within the same person as well as serum and salivary responses. The correlation of serum and CM IgG antibodies could be due to serum transudation of IgG into cervical secretions. However, evidence of IgG positive B cells in cervical mucosa [Kutteh, 1999] and the difference in IgG subclasses in serum and cervical secretions [Hocini *et al.*, 1995] indicate some local cervical IgG production. Both IgA and IgG antibodies are derived to a variable degree from systemic and local sources [Mestecky and Fultz, 1999] but detailed knowledge of their origins and their relative contribution is lacking for the female genital tract [Kutteh, 1999]. The correlation between systemic and cervical IgG within the same person has been shown to vary, and a good correlation was most evident in serum or cervico-vaginal lavage (CVL) specimens of patients with persistent cervical disease [Bontkes *et al.*, 1999].

In the present study, the cervical antibody response in women with CIN who were HPV-16 positive at the cervix, was found to be no more precise than the serum response at predicting the site of infection or the HPV association with disease. The IgG responses systemically and locally correlated best with the presence of HPV-16 DNA at the cervix. This is in agreement with previous studies [Kirnnbauer *et al.*, 1994; Wideroff *et al.*, 1995;

Bontkes *et al.*, 1999]. The IgA response though, and especially secretory IgA, is considered to be the primary effector of mucosal immunity and to be superior over IgG in eliciting defence mechanisms [Childers *et al.*, 1989; Mazanec *et al.*, 1992]. However, the importance of secretory IgA in the protection from genital HPV transmission is unknown. Both antibody classes might be important in protection against viral infection at the cervix.

In conclusion, the oral testing of HPV-specific antibodies could have important applications in the determination of HPV infection and the technique could be refined for the detection of IgG as has been demonstrated for HIV antibody testing. An oral IgG test would reflect serum IgG antibodies and could be a more type specific assay than one for IgA antibodies, as the IgG antibody responses of women with CIN in the present study in serum and CM were found more HPV-16-specific than IgA responses. The demonstration of HPV-specific IgA antibodies in oral secretions of the majority of women with CIN probably indicates that the genital mucosa operates as part of the common mucosal immune system.

CHAPTER 6. THE IMPACT OF HIV-1 STATUS ON HPV PREVALENCE AND HPV ANTIBODIES IN SERUM AND CERVICAL SECRETIONS

6.1. INTRODUCTION

There is a high prevalence of HPV infection amongst women infected with HIV-1, with a high rate of persistent infection in these women of the HPV types associated with CIN and CaCx [ter Meulen *et al.*, 1992; Sun *et al.*, 1997; Goodman *et al.*, 1999; Eckert *et al.*, 1999]. There is also an increased OR in HIV-1 infected (HIV-positive) women for the presence of cervical disease over women not infected with HIV-1 (HIV-negative women) [Mandeblatt *et al.*, 1999], with HIV-positive women showing the prevalence of a wider spectrum of HPV types, including rare types [Cappiello *et al.*, 1997]. Advanced HIV-1 disease is associated with a high prevalence, persistence and progression of CIN which is more pronounced in women with a $CD4^+$ cell count < or = 500 x 10^6/litre [Six *et al.*, 1998; Minkoff *et al.*, 1998]. It is not clear whether HIV-1 influences the pathogenesis of HPV associated disease directly through molecular interaction with HPV genes, or through its general effects on immune functions. HIV-1 infection has been shown to change the pattern of transcription of HPV E7, but is also associated with low $CD4^+$ cell count in the host [Palefsky *et al.*, 1999], so it is possible that both factors influence HPV pathogenesis [Arany and Tyring, 1998]. In support of this, triple-combination anti-retroviral therapy in women with HIV-1-related disease results in a decrease in the prevalence of CIN. There is reportedly conversion from high-grade to low-grade lesions in some patients, and complete regression in others after treatment, which is associated with a higher increase in absolute $CD4^+$ cell concentration [Heard *et al.*, 1998]. The importance of the level of immune function in the prevalence of HPV in HIV-positive women highlights its essential role in the control of HPV infection. Continued study of HPV infection in HIV-positive women should assist in the clarification of the immune mechanisms necessary for the restraint of HPV infection and its progression to cervical disease.

The findings of the Department of Health (DOH) survey in South Africa [DOH, 1999] on the prevalence of HIV-1 amongst pregnant women attending public health clinics during

the period 1 to 30 October 1998, estimated that 22.8% of women were infected with HIV-1. The highest estimates were from the KwaZulu/Natal province of 32.5% and the lowest in the Western Cape province of 5.2%. The percentage increase in the KwaZulu/Natal area over the previous year was 20.8%, with the highest increase amongst women between 15 and 19 years of age. These alarming findings are of a growing HIV-1 epidemic, especially in the KwaZulu/Natal area where a number of investigations are being conducted amongst the HIV-1 infected population. Female sex workers who are at high risk of HIV-1 infection, operating at truck stops in the KwaZulu/Natal midlands, are members of one such study.

In this Chapter, the anti-VLP-16 local cervical and systemic antibody responses of HIV-positive and HIV-negative sex workers, who are at high risk of HPV infection, will be compared in the hopes that meaningful deductions can be made on the nature of the immune response to HPV infection. The HPV-16 DNA status of the two groups of women will also be compared and related to their anti-VLP-16 systemic and local cervical antibody responses.

6.2. MATERIALS AND METHODS

6.2.1. Study population and sample collection

A joint initiative between the United Nations Programme on HIV/AIDS and the Medical Research Council of South Africa is funding an efficacy trial on a microbicide to prevent HIV-1 infection, amongst a group of female sex workers. These women operate at truck-stops in the KwaZulu/Natal midlands of South Africa [Ramjee *et al.*, 1998]. Principal investigators on this project are Doctors. Salim Abdool Karim and Gita Ramjee. The sex worker cohort also participate in a number of Medical Research Council studies, including the HIVNET 028 and Merck vaccine study, with Dr. E. Vardas as principal investigator. Informed consent was obtained from all participants. Tests revealed that 50.3% of the women were HIV seropositive (HIV-positive) by recombinant HIV-1/HIV-2 ELISA (Abbott, Chicago, USA) and Vironostika HIV Uniform II micro-ELISA 4 system (Omnimed, United Kingdom). CIN 2 was seen in 3 participants and CIN 3 in 2 participants

and all of these women with CIN were HIV positive. Vaginal infections detected in the whole group of women included *T. vaginalis, C. Albicans, N. gonorrhoeae, C. trachomatis* active syphilis and bacterial vaginosis.

From the women involved in the above study, cervicovaginal lavage (CVL) specimens and serum samples were kindly made available for use in the present study by Dr. E. Vardas and Dr. G. Ramjee (Centre for Epidemiological Research in South Africa, Medical Research Council, Durban). These samples consisted of CVL specimens from 112 members of the above group of women and paired serum samples from 83 of the 112 women collected during November and December 1998. CVL samples were collected by the insertion of 5 ml of saline into the vaginal cavity, with 1 minute wait before CVL was retrieved with a syringe. CVL samples and serum were stored at -70^0 C until required. All bloodstained samples were excluded. Of the 83 paired CVL and serum samples, 40 were from HIV-positive women and 43 from HIV-negative women. Of the 29 CVL only samples, 9 women were from HIV-positive women and 20 from HIV-negative women.

The 112 women were aged between 16 and 48 years (mean 26) and all had more than 6 sexual partners per week (mean 18.6, range: 7-40). The frequency of condom use was low. $CD4^+$ counts were available for 10 of 49 HIV-positive women, with 5 women having counts of less than 500 x 10^6/litre and none with counts less than 200 x 10^6/litre. There was no report of any CIN or CaCx in these women.

6.2.2. ELISA

The ELISAs were conducted as described in Chapter 2.2.3, with some modifications. HPV-16 VLPs used in the ELISA and were kindly supplied by MedImmune Inc. (Gaitersburg, USA). BPV-VLPs, for the assessment of ELISA background as described in Chapter 2.2.3, were supplied by Dr.Robert Rose (Rochester University, USA). All the VLPs were used at a coating concentration of 0.2μg per well or 2μg /ml (not 10μg/ml, as for previous VLP ELISAs). The CVL samples were diluted 1:1 in M-PBS and serum 1:20 in M-PBS. Total IgA and IgG estimations were performed on CVL samples to confirm the quality of the sample for assay as previously described (Chapter 5.2.3).

6.2.3. PCR

Bruce Allen and Patti Kay performed the PCR procedures on the CVL specimens. Each CVL sample was thawed, vortexed vigorously and 200µl of sample removed for DNA extraction by the method of Qiagen (Qiagen, Hilden, Germany). A portion of the CCR5 gene was amplified to assess the quality of the DNA and all CCR5 negative specimens were excluded. Amplification of the L1 region of HPV DNA was with MY primers [Manos *et al.*, 1989] and a HPV-16 specific primer [van den Brule *et al.*, 1989] was used for HPV-16 DNA amplification of all the specimens.

6.3. RESULTS

6.3.1. The influence of HIV-1 status on the local and systemic HPV antibody responses

To determine what effect the HIV-1 status of sex workers would have on their HPV antibody responses, CVL and serum samples were tested by ELISA for anti-VLP-16 IgG and IgA antibodies in HIV-positive and HIV-negative women.

6.3.1.1. VLP-16 antibodies in cervico-vaginal lavage specimens

CVL samples from 63 HIV-negative and 49 HIV-positive women were tested by ELISA for IgA and IgG antibodies to VLP-16. Boxplot analysis of the OD values obtained in the ELISA is shown in Fig 6.1. The mean OD value was higher in the HIV-positive women than the HIV-negative women for both the CVL IgA (P = 0.08) and IgG (P = 0.42) anti-VLP-16 antibodies, but these differences were not significant.

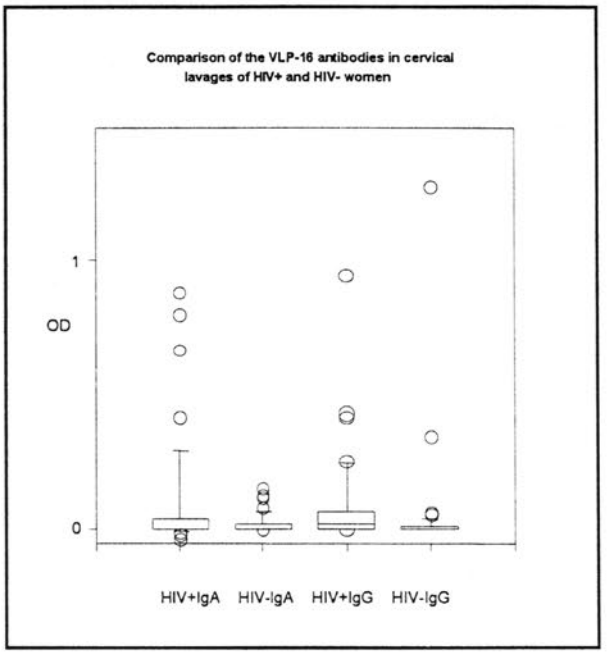

Fig 6.1. Boxplot analysis of the IgA and IgG antibody OD values (A_{492}) obtained for cervical lavage specimens of HIV-positive (HIV+) and HIV-negative (HIV-) women. Error bars represent the 10^{th} and 90^{th} percentiles. The median OD values are represented by the lines in the boxes. Mean OD values were HIV+ IgG 0.08, HIV- IgG 0.05, HIV+ IgA 0.08, HIV- IgA 0.02.

The antibody levels in CVL were generally low and in the absence of control group CVL specimens from which to estimate a cut-off value for ELISA positivity, two arbitrary cut-off levels (0.10 and 0.05) were tested as described by Wang *et al.* [1996]. Results using both cut-off levels have been presented in Table 6.1.

Table 6.1. A comparison of the IgG and IgA responses to VLP-16 in cervico-vaginal lavage specimens of HIV-positive and HIV-negative commercial sex workers using two different ELISA cut-off values

ELISA cut-off 0.05	HIV-positive Positive/total	%	HIV-negative Positive/total	%	95%CI	*P* value
VLP-16 IgA	11/49	22	9/63	14	0.71-3.49	0.3
VLP-16 IgG	16/49	33	6/63	10	1.45-8.11	0.002
ELISA cut-off 0.10						
VLP-16 IgA	11/49	22	4/63	6	1.2-10.43	0.01
VLP-16 IgG	12/49	25	3/63	5	5.14-17.23	0.002

The number of HIV-positive women with anti-IgA antibodies did not change with the two cut-off values, and the number with IgG antibodies decreased slightly with the higher cut point. In contrast, the number of HIV-negative women positive for anti-VLP-16 IgA and IgG was doubled using the lower cut point. The difference between the number of HIV-positive and HIV-negative women with CVL IgA antibodies becomes significant with the higher cut point. Regardless of the cut-off value used, there was a significant difference (P = 0.002) between the number of HIV-positive and HIV-negative women with CVL IgG antibodies. It was decided to use the lower cut point in any further assessment as the higher cut point could exclude some HIV-negative women positive for anti-VLP-16 IgG and IgA antibodies. This study found no correlation between the CVL IgA and IgG antibodies in the same sample.

6.3.1.2. VLP-16 antibodies in serum

The effect of HIV-1 status on anti-VLP-16 antibodies in serum was tested in 40 HIV-positive and 43 HIV-negative individuals. Boxplots of the ELISA OD values obtained are shown in Fig. 6.2.

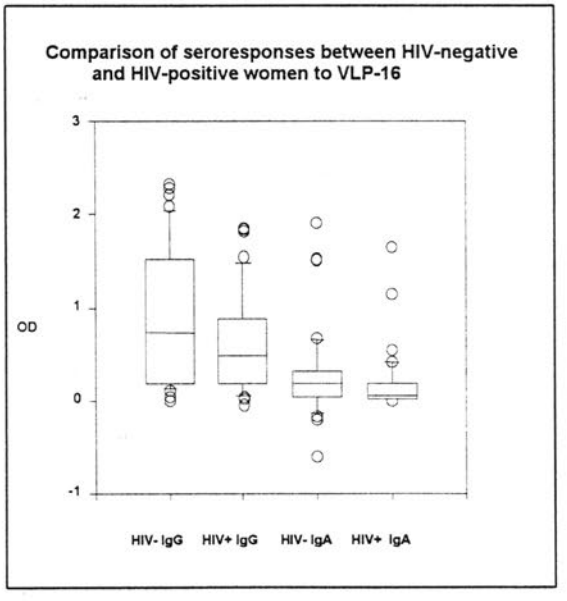

Fig. 6.2. Boxplot analysis of the VLP-16 ELISA IgA and IgG absorbances of the HIV-positive (HIV+) and HIV-negative (HIV-) women for serum samples. Error bars represent the 10^{th} and 90^{th} percentiles. The median OD values are represented by the lines in the boxes. The mean OD values were HIV+ IgG 0.63, HIV- IgG 0.90, HIV+ IgA 0.17 HIV-IgA 0.26.

The mean OD values for both serum anti-VLP-16 IgG and serum IgA were higher in the HIV-negative women as compared with the HIV-positive women. These differences were significant for IgG ($P = 0.04$) but not for IgA ($P = 0.26$).

The number of women positive for anti-VLP-16 IgA and IgG antibodies was estimated using ELISA cut-off values estimated from children's sera as described in Chapter 2.2.4. The number of women positive for anti-VLP-16 IgA and IgG are shown in Table 6.2.

Table 6.2. A comparison of the anti-VLP-16 antibody prevalence in serum of HIV-positive and HIV-negative sex workers

Test	HIV-positive		HIV-negative		*P* value
	n/total	%	n/total	%	
Serum IgG	27/40	68	30/43	70	0.82
Serum IgA	6/40	15	17/43	40	0.012

There was a significant difference between the number of HIV-negative women positive for anti-VLP-16 serum IgA (17/43) compared with HIV-positive women (6/40, $P = 0.012$). There was no significant difference between the number of women with anti-VLP-16 serum IgG antibodies in the HIV-positive group (27/40) and HIV-negative group (30/43, $P = 0.82$).

6.3.1.3. HPV DNA prevalence in HIV-negative and HIV-positive women

To establish the effect of HIV-1 status on HPV infection, the presence of HPV DNA was determined on a group of 99 sex workers (47 HIV-positive and 52 HIV-negative) and 62 were found positive for HPV DNA of any HPV type. HPV-16 typing was available on 57 samples of the total 62 of HPV DNA positive specimens (Table 6.3).

Table 6.3. The HPV DNA status of HIV positive and HIV negative sex workers and the number positive for HPV-16

Test	HIV-positive		HIV-negative		P value
	n/total	%	n/total	%	
HPV DNA (any type)	40/47	85	22/52	42	0.00001
HPV-16 DNA	5/38	13	1/19	5.2	0.36

There was a significantly higher prevalence of HPV DNA in HIV-positive women than in the HIV-negative women (P = 0.00001). Multiple regression analysis to determine the role of possible confounders (years as a sex worker, number of partners per day, condom use, gonorrhoea infection, syphilis infection and HIV-1 sero-positivity), on the significance of such a high HPV DNA prevalence in the HIV-positive women, found the only significant variable associated with HPV infection was HIV-1 sero-positivity *(P* = .0347). This indicates that HIV-1 alone influences HPV infection with or with out the other factors. Five HIV-positive women and one HIV-negative woman were positive for HPV-16. Despite the low level of HPV-16 infection detected, a large number of HIV-positive (68%) and HIV-negative (70%) women had serum anti-VLP-16 IgG antibodies and 40% of HIV-negative women serum IgA.

It was of interest to note the prevalence of anti-VLP-16 antibodies in CVL and serum of those women who were HPV-16 DNA positive. The anti-VLP-16 antibody presence in CVL and serum of the HPV-16 DNA positive sex workers (5 HIV-positive and 1 HIV negative) is shown in Table 6.4.

Table 6.4. The anti-VLP-16 antibody presence in CVL and serum of the HPV-16 positive sex workers

Sex worker		CVL IgA	CVL IgG	Serum IgA	Serum IgG
HIV-positive	1	-	-	-	+
	2	-	-	-	+
	3	-	-	-	+
	4	-	-	-	+
	5	-	-	-	+
HIV-negative	1	+	-	+	+

+ = antibodies present, - = no antibodies.

The HIV-positive women, who had detectable HPV-16 DNA, appeared only able to mount a detectable serum anti-VLP-16 IgG response. There was no correlation between CVL anti-VLP-16 IgG or IgA antibodies and HPV-16 DNA status in the HIV-positive women as no HPV-16 positive women who were HIV-positive, had anti-VLP-16 CVL antibodies. The 1 HIV-negative, HPV-16 DNA positive woman had anti-VLP-16 CVL IgA, but not IgG and both serum IgA and IgG anti-VLP-16 antibodies. Irrespective of HIV status, all

the HPV-16 DNA positive women had anti-VLP-16 serum IgG antibodies but none of the HIV-positive women had a corresponding serum anti-VLP-16 IgA.

6.3.2 Comparison of HIV-positive and negative sex workers with women with CIN

To determine the possible differences in antibody responses systemically and locally and in HPV-16 DNA status, HIV-positive and HIV-negative sex workers were compared with women with CIN (Chapter 5) with regards HPV-16 DNA presence and their serum and cervical antibodies to VLP-16. The results are shown in Table 6.5.

Table 6.5. The comparison of the number of HIV-positive and -negative women and women with CIN with serum and cervical antibodies to VLP-16 and their HPV-16 status

Test	HIV-positive		HIV-negative		CIN	
	No positive/total	%	No positive/total	%	No positive/total	%
HPV-16 DNA	5/38	13	1/19	5.2	10/27	37
Cervical VLP-16 IgG	16/49	33	6/63	10	10/26	38
Cervical VLP-16 IgA	11/49	22	9/63	14	11/26	42
Serum VLP-16 IgG	27/40	68	30/43	70	43/82	52
Serum VLP-16 IgA	6/40	15	17/43	40	37/82	46

This comparison was able to highlight some differences in the three groups of women with regard to their HPV prevalence and antibody responses to VLP-16. The most striking difference was the much higher prevalence of HPV-16 DNA in women with CIN as compared with the HIV-positive and HIV–negative women. More HIV-positive women displayed a local antibody response compared with HIV-negative women, particularly for cervical IgG, which was comparable with the number of women with CIN with cervical IgG responses. More HIV-negative and HIV-positive sex-workers demonstrated serum IgG responses than women with CIN, whereas few HIV-positive women had serum IgA antibodies compared with HIV-negative women and women with CIN. More women with CIN mounted an antibody response at the cervix than the sex-workers, irrespective of their HIV status.

6.4. DISCUSSION

The aims of this study were to evaluate the antibody responses systemically and locally in HIV-positive sex workers and relate them to the responses of HIV-negative sex workers in the hopes that this would give insights into the normal response to HPV infection in immuno-competent individuals. The nature of the lifestyle of the commercial sex-workers studied, with between 7 and 40 partners per week, resulted in a high level of exposure to HPV. This was demonstrated in this study by the high rate of HPV infection found in the HIV-positive sex workers (85%) and is consistent with findings reported by others [Sun *et al.*, 1997; Cappiello *et al.*, 1997]. The high rate of HPV infection in HIV-positive women, can be related to their $CD4^+$ count [Sun *et al.*, 1997] with women with a $CD4^+$ count less than 500 x 10^6/litre, showing a greater prevalence of HPV infection than those with higher $CD4^+$ counts. In the present study $CD4^+$ counts were only available for 10 HIV-positive women, 5 counts less than 500 x 10^6/litre, too few to be of use in comparative evaluations. Despite their high-risk lifestyle and large number of sexual partners, only 42% of the HIV-negative sex workers were HPV DNA positive at the cervix, compared with the 85% of the HIV-positive women. This could indicate that the immunocompetent HIV-women were better able to cope with the high rate of HPV exposure (due to their large number of sexual partners) than the HIV-positive women. There was no difference in the number of sexual partners or in condom usage between HIV-negative women and HIV-positive women, so there was no difference in the rate of HPV exposure in the two groups.

In order to determine how the immunocompetent, HIV-negative women were more successful in their clearance of HPV infection, the antibody responses to VLPs of HPV-16 at the cervix and systemically were compared in the HIV-positive and HIV-negative women. Antibodies to HPV in CVL samples of HIV-positive women have not been previously reported. This study found that more HIV-positive women were able to mount both an anti-VLP-16 IgA and IgG response at the cervix than the HIV-negative women and this was significantly so for IgG antibodies ($P = 0.002$). Interestingly the mean OD values obtained in the CVL IgG ELISAs was significantly in higher in the HIV-negative women ($P = 0.04$) but not significantly higher for the IgA OD values. The relevance of this is unclear and the response was not quatitated to confirm higher antibody titres in the HIV-negative women. In assessing the presence of anti-HIV antibodies in HIV-positive

individuals, IgG antibodies have been shown to predominate over IgA [Lu *et al.*, 1993; Hocini *et al.*, 1997] and the overproduction of HIV-specific IgG in CVL contrasts with a normal or impaired anti-HIV IgA local response [Belec *et al.*, 1995]. This was confirmed in the present study for HPV antibodies, with more HIV-positive women having anti-HPV IgG antibodies in CVL and higher levels of IgG compared with IgA antibodies. The local mucosal immune response in HIV-positive women has reportedly been shown to differ from HIV-negative women [Bardeguez *et al.*, 1997]. Local immunity is markedly enhanced in the non-auto immune deficiency stage of the disease [Lu *et al.*, 1993]. It has been postulated that a systemic polyclonal B-cell activation, characteristic of HIV infection, could affect the secretory immune system at the cervix and increase local IgA and IgG [Lane *et al.*, 1983]. HIV-positive women also have an increased local mucosal lymphocyte (mainly $CD3^+CD8^+$) presence, with over 80% of the $CD3^+CD4^+$ cells being memory cells. This is reportedly indicative of a local immune response elicited in these women at the entry infection site, to protect against further infection [Bardeguez *et al.*, 1997]. These lymphocytes could modulate the local IgA and IgG response at the genital mucosal surface [Bardeguez *et al.*, 1997].

Both anti-HIV IgA and IgG antibodies have been shown to impair virus transmission across the cervico-vaginal mucosa, with IgA being more efficient [Hocini *et al.*, 1997]. In HIV-positive individuals, both mean IgG and IgA anti-HIV antibody titres have been shown to be higher than that of serum indicating an increased local synthesis of both isotypes [Belec *et al.*, 1995]. It would appear therefore from the present study, that HIV-positive women have an improved local antibody production (although IgA not significantly) and by inference possibly also an improved protection of the local mucosa against HPV infection. All the sex workers had other STDs and other vaginal infections, so local inflammation caused by these could not be a contributing factor. The local HPV antibody response (as indicated by anti-VLP-16 antibodies) of HIV-positive women does not appear to be relevant to their increased HPV infection rate, but could relate to some other aspect of their HIV-disregulated immune response.

The majority of sex workers (HIV-positive, 68% and HIV–negative, 70%) had anti-VLP-16 serum IgG antibodies. Systemic IgG levels are reportedly not related to the clearance of HPV [Bontkes *et al.*, 1999] but indicate a persistent infection [Remmink *et al.*, 1995]. Serum IgG antibodies can be indicative of lifetime exposure to HPV-16 [Olsen *et al.*,

1997], and serum IgG antibodies to VLP-16 are also related to the number of sexual partners of an individual and increase by 4% per increase in partner [Dillner *et al.*, 1996]. All the women under study had between 7 and 40 sexual partners per week with an insignificant difference between the average partners per week of the HIV-negative women (18.3 partners per week) compared with an average of 18.7 for the HIV-positive women. Their large number of sexual partners probably accounts for the large percentage of HIV-positive and HIV-negative women with serum anti-VLP-16 IgG.

Serum antibody responses to HPV antigens (VLP-6 and VLP-16) have been reported in HIV-positive homosexual men with high-grade anal squamous intraepithelial lesions [Hagensee *et al.*, 1997]. The presence of anal warts corresponded with anti-VLP-6 antibodies in the HIV-positive and HIV-negative homosexual men and the anti-VLP-16 antibodies with high-grade anal intraepithelial lesions. In the present study no women presented with cervical lesions, but more HIV-negative women were shown to mount an anti-VLP-16 serum antibody response than HIV-positive women and this was significantly so for the IgA response ($P = 0.012$). This is supported by the finding that the serum IgA antibody response to HIV-1 (in HIV-1 infected individuals) is poor, compared with the IgG response [Belec *el al.*, 1995]. From these results it would appear that it could be an anti-HPV serum response and possibly the serum IgA response that was responsible for the HIV-negative sex workers ability to cope with high HPV-16 exposure, and not their local antibody response. These women were able to clear HPV-16 infection with a resultant reduced HPV-16 DNA prevalence, compared with the HIV-positive women. Systemic anti-VLP-16 IgA levels have been shown to correlate with viral clearance in infected individuals [Bontkes *et al.*, 1999]. Although it is unclear how systemic antibodies function to eliminate virus, it is proposed that the relatively few HIV-positive women with anti-VLP-16 serum IgA in the present study (6/40), could be indicative of an increased risk of in cervical disease in these women. The HIV- positive women who had detectable HPV-16 DNA only had a serum anti-VLP-16 IgG response and no CVL anti-VLP-16 antibodies or serum IgA. It could be postulated that the reason they acquired HPV-16 was the lack of local antibodies to clear the virus. These women were in the group (32/40) with no anti-VLP-16 serum IgA antibodies and could be at increased risk of HPV-16 associated cervical disease.

The comparison of the HPV-16 prevalence (antibodies or DNA) in the HIV-positive and HIV-negative sex workers with the CIN patients confirmed the elevated HPV-16 prevalence in women with cervical disease [Lorincz *et al.*, 1992]. Anti-VLP-16 serum IgG was more prevalent amongst the sex workers than the women with CIN and probably related to their number of sexual partners. Although only a few sex workers were HPV-16 DNA positive the anti-VLP-16 serum IgG antibodies in 68%-70% of them, could also be indicative of a transient infection [Wikström *et al.*, 1995] or a previous infection. The few HIV-positive women with anti-VLP-16 serum IgA could relate to the high level of HPV infection in these women and could be considered an indication of a poor prognosis for the development of cervical diseases [Bontkes *et al.*, 1999]. HIV infection does increase the risk of CIN in those with HPV infection [Cappiello *et al.*, 1997]. More women with CIN in this study were shown to mount an anti-VLP-16 antibody response at the cervix. The significance of this is unknown but could relate to the fact that more of the women with CIN were HPV-16 DNA positive at their cervical lesion. It could also be due the fact that CM was assayed in these women as opposed to the CVL of the HIV-positive and –negative women, where the antibody levels would be lower because of the larger dilution factor. It will be important to compare the relevant efficacy of these two methods of cervical antibody sampling, before firm conclusions can be drawn.

Finally, in conclusion it would appear that it is an effective serum IgA response that is indicative of the ability of the HIV-negative sex-workers to prevent HPV-16 infection despite their high levels of exposure to HPV-16.

CHAPTER 7. HUMAN PAPILLOMAVIRUS TYPE-16 VIRUS-LIKE PARTICLES AND L1-16/RECOMBINANT BCG AFFORD PARTIAL PROTECTION IN MICE AGAINST CHALLENGE WITH RECOMBINANT VACCINIA VIRUS EXPRESSING THE L1 GENE OF HPV TYPE 16

7.1. INTRODUCTION

An effective, affordable HPV vaccine could significantly reduce the more than 500000 new CaCx cases diagnosed worldwide each year, 80% of which are in the developing world [Bosch *et al.*, 1995]. The development of an HPV vaccine has been significantly hindered by the difficulty of HPV propagation in culture [Lowy *et al.*, 1994]. However, HPV virus-like particles (VLP) produced by baculovirus or vaccinia virus (Vv) expression in cells [Kirnbauer *et al.*, 1992; Hagensee *et al.*, 1993; Rose *et al.*, 1993], are emerging as immunogens with great potential as prophylactic HPV vaccines [Kirnbauer, 1996a]. External conformational structures on VLPs define neutralising epitopes. These are important in the natural immune response to HPV, since seroreactivity in humans is directed mainly against conformational epitopes on virions and VLPs [Bonnez *et al.*, 1991; Kirnbauer *et al.*, 1994a; Rose *et al.*, 1994a; Wang *et al.*, 1997; Sasagawa *et al.*, 1998]. Several studies have shown that PV VLPs elicit high titres of neutralizing antibodies in serum and protection from experimental challenge with infectious virus in animal papillomavirus models [Breitburd *et al.*, 1995; Kirnbauer *et al.*, 1996b; Rose *et al.*, 1994a; Suzich *et al.*, 1995]. Immunisation of cottontail rabbits with VLPs composed of the cottontail rabbit papillomavirus (CRPV) L1 major capsid protein has recently been shown to protect rabbits against CRPV challenge (Breitburd *et al.*, 1995; Christensen *et al.*, 1996; Jansen *et al.*, 1996). In contrast rabbits vaccinated with disrupted CRPV-VLP or BPV-VLP developed papillomas, suggesting that protection was type-specific and dependent on conformational epitopes on the surface of the VLP or whole particles to elicit the correct response. It also appears that most antibodies in human sera are type specific and react to intact VLP recognising the same or closely related major antigenic determinants [Wang *et*

al., 1997]. In a canine oral papillomavirus model, neutralising antibodies have been shown to confer protection from infection with canine oral papillomavirus [Suzich *et al.*, 1995]. A tumour cell challenge model reported by De Bruijn *et al.* [1998] has demonstrated the therapeutic effect of HPV-16 VLP (VLP-16) and Greenstone *et al.* [1998] the therapeutic effect of chimeric HPV-16 L1/L2/E7 VLP. There has also been good progress in the understanding of the role of CMI in the control of papillomavirus infections and the induction of HPV-specific CTL and Th responses are thought to be an essential prerequisite for an HPV vaccine. Several clinical and experimental studies have shown that CMI responses play a role in both susceptibility to and regression of HPV infections. Immunocompromised individuals show a rapid progression of genital HPV [Alloub *et al.*, 1989; Frazer *et al.*, 1986; Petry *et al.*, 1994]. Strang *et al.* [1990] demonstrated proliferative T cell responses in normal healthy individuals to peptides derived from protein sequences of HPV-16 L1 and E6. As discussed in Chapter 1.11.3. the understanding of T cell immunity has been assisted by the identification of MHC class I and II epitopes of HPV proteins [Altman *et al.*, 1992] and also the CTL epitopes [Feltkamp *et al.*, 1993]. Kast *et al.* [1994] have identified several high affinity binding peptides of HPV-16 E6 and E7 proteins for human HLA-A allelles. The loss of MHC class 1 expression and the reduction of proliferative T cell responses to HPV antigens have been shown in women with CaCx [Gill *et al.*, 1998; Luxton *et al.*, 1996; 1997; Conner and Stern, 1990]. Several studies in mice have shown that VLPs are also capable of priming a productive CMI response [Zhou *et al.*, 1991]. This response is associated with the production of Th1-type cytokines and of delivering of HPV antigens to the HLA class I processing pathway for priming of cytolytic $CD8^+$ T cells [Peng *et al.*, 1998; Greenstone *et al.*, 1998; Dupuy *et al.*, 1997]. A similar HPV-16 L1-specific T cell response has also been demonstrated in humans [Rudolf *et al.*, 1999].

HPV vaccine development has been further impaired by the inability to infect laboratory animals with HPV because of their specific host tropism. This has abrogated the demonstration of protection against HPV challenge. This chapter describes the development and use of a recombinant HPV-16 L1-vaccinia virus challenge model in mice to demonstrate protection in VLP-16 vaccinated mice and to investigate the importance of CMI induced in the mice. This challenge system was selected, as the clearance of recombinant Vv (rVv) is dependent on vaccine-induced T cells recognising the gene expressed by rVv

Recombinant vaccinia virus is a popular laboratory expression system because of several favourable properties. The system exhibits retention of infectivity, thermostability, the ability to grow high titre viral stocks, the wide host range of the virus and the large capacity for foreign DNA and the rapid and high level of gene expression [Moss, 1996]. The naked Vv DNA is not infectious and so rVv genomes are formed by homologous recombination with the gene of interest in a plasmid insertion vector in Vv infected cells [Mackett *et al.*, 1982]. The foreign gene is expressed by engineering its coding sequence downstream of a Vv promoter. This is necessary as the virus encodes its own DNA-dependent RNA polymerase, which does not recognise promoters other than those of poxviruses. For the present model it was necessary to express the inserted L1 gene from an early promoter. Expression of L1/L2 from rVv off late promoters produces both systemic and mucosal antibodies in mice [Hagensee *et al.*, 1995]. Antibodies have been elicited from foreign genes from both early and late Vv promoters, but late promoters fail to elicit CTL responses due to possible down regulation of MHC antigens after Vv infection [Coupar *et al.*, 1986; Zhou *et al.*, 1991]. Alternatively, interference of the proper processing of foreign antigens for presentation to T cells by inhibition of cellular proteases late in Vv infection [Smith *et al.*, 1989] might be the reason late promoters fail to elicit T cell responses.

Bronte *et al.* [1997] have demonstrated that dendritic cells infected with rVv encoding a gene under a late promoter failed to activate specific CTL, whereas under an early promoter CTL were activated. The promoter used in the present study is the constitutive, synthetic pE/L which has been successfully used for the efficient expression of foreign genes [Chakrabarti *et al.*, 1997] and which has early and late functions. The Vv site chosen for the insertion of the L1 gene was the non essential thymidine kinase (TK) gene which allows genetic selection of TK^- recombinant virus by plaque assay on TK^- cells in the presence of 5- bromodeoxyuridine (BUdR). The incorporation of the E coli β-galactosidase (β-gal) gene into vaccinia virus with the L1 gene allows for visual distinction between rVv and spontaneous TK^- mutants [Chakrabarti *et al.*, 1985]. The chromogenic substrate used for the detection, 5-bromo-4-chloro-3-indoyl-β-D galactosidase (X-gal), is converted by β-gal into a deep blue colour.

Binder and Kündig [1991] first described the use of rVv used as a challenge system where it assisted in the assessment of the respective roles of antiviral protection by $CD8^+$ and

$CD4^+$cells. That study used two groups of mice, a CTL non-responder mouse strain which cannot mount a CTL response to vesicular stomatitis virus (VSV) and a responder mouse strain that generate MHC-restricted CTL specific for the nucleoprotein (NP) of VSV. The responder mouse strains, primed with VSV, were shown to elicit a measurable CTL response to, and were protected from, challenge with rVv expressing the NP of VSV. This response was susceptible to $CD8^+$ T cell depletion, but not $CD4^+$ depletion or anti-IFN-γ treatment. The VSV-primed non-responder mice did surprisingly, mount a CTL against rVv expressing VSV NP, but the response was susceptible to $CD4^+$ depletion and anti-IFN-γ treatment and not $CD8^+$ depletion. Challenging with large viral doses caused the death of non-responder mice after rVv challenge but not in the responder mice and the conclusion was that $CD4^+$ T cell-dependent IL antiviral protection was weak in comparison with the CD8-mediated CTL protection. Bachmann *et al.* [1994] used rVv expressing the nucleoprotein of vesicular stomatitis virus (VSV) to investigate the efficacy of a potential viral protein vaccine. They were able to show that mice primed with three different recombinant viral proteins from a baculovirus expression system, the glycoprotein and NP of lymphocytic choriomeningitis virus (LCMV) and the NP of VSV, induced CTL which protected respective mice from challenge with three rVv expressing the different proteins. This technique has also been applied to assess HIV type 1 vaccine regimes in mice [Belyakov *et al.*, 1998; Kent *et al.*, 1998]. Tests were able to show that mice immunised rectally with an HIV-1 peptide vaccine were protected from intrarectal challenge with rVv, expressing the HIV-gp160 protein [Belykov *et al.*, 1998] and that protection was by mucosally and systemically induced CTL. Kent *et al.* [1998] used DNA immunisation of mice with plasmid DNA expressing *env* and *gag* genes of HIV-1 and booster immunisation with recombinant fowlpox virus expressing these genes, to elicit protection against challenge with rVv expressing *gag* and *env*.

The use of recombinant Vv challenge allows selective evaluation of cell mediated rather than neutralizing antibody immune mechanisms, because Vv recombinants do not express the transfected gene products in their envelope in a form directly accessible to antibodies [Zinkernagel *et al.*, 1990]. The WR (Western Reserve) strain of Vv was used, which causes disseminating infection in mice with the highest titres of virus being measured in the ovaries [Binder and Kundig, 1991]. The importance of CMI induced by VLP-16 vaccination has been demonstrated [Marais *et al.*, 1999]. That study showed that

immunisation with VLP-16 does elicit a protective T cell response characterized by a Th1 cytokine profile and with some Th2 IL-2 production, which is capable of protecting mice against challenge with recombinant HPV-16 L1-Vv (VvL1$_R$-16). The practical application of this challenge model in the assessment of the protection inducing capabilities of a potential BCG vaccine and that of a potential VLP oral vaccine will be presented.

The human tuberculosis vaccine, Bacille Calmette-Guerin (BCG), employing *Mycobacterium bovis*, has a number of features that make it attractive as a live vaccine vehicle. BCG is a highly effective adjuvant, and has been administered to almost 3 billion individuals including infants with a low incidence of side effects [Lotte *et al.*, 1984]. Vaccination requires a single dose to induce a long lasting immunity [Flynn 1994] elicited by a long-term systemic, protective IgG response as well as S-IgA response against a target pathogen [Langerman, 1996]. Recombinant BCG (rBCG), expressing proteins from various infectious agents have been described, inducing both antibody and T cell, protective immune responses in laboratory animals [Aldovini and Young, 1991; Langranderie *et al.*, 1997; Stover *et al.*, 1993]. This laboratory is investigating the development of rBCG for HPV L1 expression as a potential HPV prophylactic vaccine. L1-16 ORFs were introduced into two *Mycobacterium* shuttle vectors under the control of heat-shock protein, *hsp*60 promoters and subsequently L1-16 cloned into BCG. The resulting constructs will be examined for their efficacy in affording protection against challenge with rVv expressing L1-16.

Oral immunisation of mice by gavage with VLPs, have shown the induction of serum anti-VLP IgG and IgA, which were neutralising [Rose *et al.*, 1999]. The oral route of immunisation is attractive because of the ease of administration. It is important to establish whether this vaccination route for VLP will induce a CMI response in addition to the humoral one. This chapter will examine the protective cell mediated immune responses induced by potential HPV-16 L1 vaccines in a mouse model. These will be assessed by intraperitoneal (i.p.) immunisation of mice with VLP-16, rBCG expressing the HPV-16 L1 protein and VLP-16 by intestinal gavage and the subsequent challenge of the mice with rVv expressing the HPV-16 L1.

7.2. MATERIALS AND METHODS

7.2.1. Virus-like particles (VLPs)

VLPs were produced in a baculovirus expression system [Rose *et al.*, 1994b). The VLP-16 preparation was provided by Dr. R. Rose (Rochester University, Rochester, USA).

7.2.2. The construction of vaccinia virus recombinants

The L1 gene of HPV-16 (Rochester–1K/UR2 strain; HPV-$16_{Rochester-1K/UR2}$) which was obtained from W. Bonnez (Rochester University, Rochester, USA) [Bonnez *et al.*, 1998], had been cloned as described in Rose *et al.* [1993; 1994b] and White *et al.* [1998]. This L1 gene (designated $L1_R$) in the construct $pVL1_{Rochester}$, was cloned into the Sma 1 site of pMos blue. The L1 gene was further subcloned into the Sal 1/Sma 1 sites of the Vv shuttle vector, pSC65 (B. Moss NIH), downstream of the synthetic early/late promoter (PE/L), generating pSC65L1 $_R$–16 (James Maclean is acknowledged for the final construct). This vector introduces the L1 gene into the TK site of Vv. The resultant recombinant Vv is TK^-. In the presence of the thymidine analogue, BUdR, which is only phosphorylated by an active TK, BudR is incorporated lethally into the nonrecombinant TK^+ virus, leaving only TK^- rVv viable. The plasmid also contains the β-galactosidase gene for the visual selection of blue recombinant plaques [Chakrabati *et al.*, 1985]. Spontaneous TK^- mutants do frequently occur but these will not express the β-galactosidase gene and will form normal, uncoloured plaques as opposed to the blue rVv plaques when stained with the chromogenic substrate, Xgal (Biosolve B.V. Netherlands).

Transfection with a linear plasmid was found to increase the efficiency of the transfection events by ensuring that double crossover recombination is achieved with Vv [K. Dumbell, personal communication]. Single-crossover events are unstable and revert to frequently wild type [Spyropoulos *et al.*, 1988]. Recombinant $VvL1_R$-16 is detected by X-gal stained blue cells.

7.2.3. Vaccinia virus

Virus stocks,Vv (strain WR; from Dr John Williamson, St Mary's Hospital, London) and rVv were prepared from the chorio-allantoic membranes (CAMs) of 12 day old, embryonated hen's eggs [Westwood *et al.*, 1957] and purified by a modification of the method of Joklik [1962]. CAMs were harvested 3 days post infection (p.i.) and placed in a bottle containing 1ml per membrane of a 3:1 mixture of McIlvain's (MI) buffer (4mM citric acid, 0.2M Na_2HPO_4 12 H_2O, pH 7.4) and the organic solvent Arklone-X (1,1,2-trichloro-1,2,2-trifluoroethane) and 2mm glass beads. The suspension was shaken vigorously for 2 min to disrupt the CAM tissue and release virus particles. After brief centrifugation at 600g, the supernate was harvested and the tissue pellet once more treated to the extraction process. Both supernates were pooled and the virus semi-purified by centrifugation through 36% sucrose. The final pellets were suspended in MI buffer.

7.2.4. Cell cultures

Standard techniques were used. African green monkey kidney (CV-1) and human TK^- 143B (TK^-) cells were grown at 37 ^{0}C in a 5% CO_2 incubator in Dulbeccos minimum essential medium (DMEM, Highveld Biologicals) containing 10% fetal bovine serum. In the case of the TK^- cells, 50μg/ml 5-bromodeoxyuridine (BUdR) was included.

7.2.5. Antisera

Rabbit antisera raised against BPV-1 particles were obtained from Dako, (Dako, Denmark). Dr Robert Rose supplied rabbit antisera (079) raised against intact VLP-16.

7.2.6. Transfections

Standard molecular biology techniques as described by Sambrook *et al.*,. [1989], were used for the production of plasmid DNA and purification was by Nucleobond (Macherey-Nagel, Düren, Germany). Transfection was with linear plasmid DNA, to ensure stable, double crossover recombination events. The plasmid pSC65 $L1_R$ was digested with ScaI, which

cuts once in the ampicillin gene only. A 6 well tissue culture plate (Nunc, Denmark) with a monolayer of CV-1 cells was infected with Vv (WR) at a moi of 0.1. One hour later the virus was removed and the cells were transfected with linear pSC65 $L1_R$, plasmid DNA using DOTAP (Boehringer Mannheim, Germany) according to the manufacturer's instructions. Briefly, 10μg linear plasmid in 200μl Hepes-buffered saline (HBS) was mixed with 60μl DOTAP made up to 200μl with HBS for 10-15 min at room temperature. The virus inoculum was removed from the CV-1 cells and replaced with DMEM plus 4% fetal calf serum (4% DMEM) and 100μl of the transfection mix per well. The infection was allowed to progress for 2 days and then cells were stained with X-gal to visualise the rVv infected cells. Recombinant virus was isolated as described by Macket *et al.* [1984]. Cells infected with rVv were dislodged into the medium with a rubber policeman, centrifuged briefly and the medium discarded. The cells were resuspended in 200μl DMEM and subjected to 3 cycles of freeze-thawing using dry ice with ethanol and a water bath at 37 ^{0}C. TK^- cells in 6–well plates, were infected with various dilutions of the cell lysate, left for one hour before the inoculum was removed and replaced with an 1ml agarose overlay. This consisted of a 2% low melting agarose (SeaPlaque FMC Bio Products) plus an equal volume of 2x concentrated DMEM with 1/100 volumes of 0.5mg/ml BUdR at 42 ^{0}C. The following day, the cells received a second agarose overlay as before but a 2ml overlay/well with the addition of X-gal (1/150 volumes of 50mg/ml stock in DMSO). Resultant blue plaques were picked into a small quantity of DMEM and the cells frozen and thawed 3 times. Recombinant Vv was subjected to several rounds of selection and plaque purification and amplification in TK^- cells until sufficient, pure recombinant was obtained. The rVv-L1 was designated $VvL1_R$-16 and was propagated in fertilised eggs as described. Viral titre was determined by infection of 10 fold dilutions of virus on CV-1 cells in 24-well plates. Two days post infection, viral plaques were visualised after staining cells with Ziehl Neelsen, carbol fuchsin stain.

7.2.7. Immunofluorescence

To establish whether there was expression of the L-16 gene from rVv, the $VvL1_R$-16 infected CV-1 cells were examined by immunofluoresence with antisera both to intact VLP-16 (B. Rose, 079 antiserum) and the polyclonal BPV-1 antiserm. CV-1 cells were grown on 19mm coverslips and infected at 30pfu/cell. The cells were left for one day and

then fixed in cold acetone for 10 min at 4^0C and blocked with 2% ovalbumen in PBS for 20 min at room temperature, to prevent non-specific antibody binding. Antisera were added, diluted 1:200 in 2% ovalbumen/PBS and incubated for 1hour at 37^0 C. After rinsing in PBS twice for 10 mins, fluorescene immuno-tagged conjugated (FITC) anti serum was applied (1:40 dilution) for 30 mins at 37^0 C. The cells were further rinsed twice in PBS and once in distilled water, before air drying and mounting on a glass slide. The cells were examined under a fluorescent microscope.

7.2.8. VLP-induced protection as assayed in a mouse challenge model

7.2.8.1. The assessment of protection against $VvL1_R$-16 challenge

Inbred mouse strains, BALB/c or C57BL/6, (Animal Unit, University of Cape Town) were used for this assay. In the initial experiment, two groups of four female BALB/c mice (8-12 weeks old) were compared. Group A was vaccinated with 10ug of VLP-16 in PBS plus 10% insect cell extract (ICE) [Bachmann *et al.*, 1994] and Group B was left unvaccinated. After thirteen days groups A and B were both challenged with $VvL1_R$-16 (5×10^6pfu). Five days after challenge the mice ovaries were harvested. The ovaries of each mouse was chopped finely, placed in McIlvains buffer (4mM citric acid, 0.2M $Na_2HPO_4.12H_2O$) (100mg ovary/ml) and homogenised using a ten broeck grinder (30-40 strokes). This was followed by three cycles of freeze thawing and then preparations were centrifuged at 2000 rpm for 10 minutes to pellet cell debris. The supernatant fluid was titrated in CV-1 cells and the $VvL1_R$-16 titre per mouse calculated after visualising plaques on the cell monolayers with carbol fuchsin stain. The limit of detection was 10^3 pfu. To determine that rVv was maintained after challenge in the mice and that there had been no reversion to wild type, CV-1 cells infected with ovarian extracts were overlaid with X-gal stain to reaffirm the presence of recombinant virus.

A similar experiment was conducted using C57B/L6 mice as a model system. Three mice were vaccinated with 10µl VLP-16 in 90µl PBS plus 10% ICE. Three mice were left unvaccinated and all 6 mice challenged as before, after 13 days. . All 6 ovaries from each group were combined for virus extraction and titration because of the small difference in titre found between individual BALB/c mice ovaries.

7.2.8.2. The assessment of protection against Vv wild type

It was important to establish the effect of challenge with wild type Vv on VLP-16 vaccinated mice and those that were unvaccinated. A group of 3 mice were vaccinated i.p. with 10μg of VLP-16 and 3 mice were left unvaccinated as controls. After 13 days all 6 mice were challenged with Vv wild type (5 x 10^6 pfu/mouse in PBS) and 5 days later the ovaries were removed. All 6 ovaries from vaccinated mice were combined, as were those from control mice. Virus was extracted and titrated as described in 7.2.8.1.

7.2.8.3. The evaluation of potential rBCG vaccines

The 3 BCG constructs were provided by James Maclean, (University of Cape Town Medical School).

1) pMV361-L1n rBCG, expressed the truncated $L1_{Rochester}$-16 protein, with a truncation removing 66 base pairs from the carboxy-terminal, which would remove the nuclear localisation signal (NLS).

2) pNIV-$L1_{tr}$ rBCG, expressed a truncated L1-16 protein with a stop codon at Cys428 caused by a PCR artifact, and was also presumed to have no NLS. This L1 protein had been cloned from a CaCx patient in this laboratory (W Burgers, MSc thesis, 1996).

3) pNIV-L1 rBCG, expressing a full-length $L1_{Rochester}$-16 protein.

All three constructs were capable of chromosomal integration.

Seven groups of three mice were used in the experiment. Four groups of three BALB/c mice were inoculated i.p. with 100μl rBCG ($2x10^6$). One control group of mice was vaccinated with wild type BCG. Two groups of mice (1 vaccinated with pNIV-L1 rBCG and 1 unvaccinated) were challenged with Vv wild type and the remainder of the mice challenged with $rVvL_R$-16. For the ovarian titration all the ovaries from each group were combined.

7.2.8.4. The evaluation of an oral VLP-16 vacccine

Three groups of 5 female BALB/c mice were fed by gastric lavage three different concentrations of VLP-16 (100μg, 50μg, and 10μg per mouse). This was administered on

three occasions at two weekly intervals. One group of 5 mice received no oral vaccination. After 6 weeks, mice were all challenged with rVvL$_R$-16 ($5x10^6$pfu/mouse). Five days later ovaries were removed. The ovaries from all the mice in each group were combined, the virus extracted and virus titre determined on titration plates.

7.3. RESULTS

7.3.1. Immunofluorescent staining

The rVv expressing the L1 protein was assessed for the presence of the expressed protein in rVv infected CV-1 cells. Immunofluorescent staining for the expressed L1 gene in CV-1 cells revealed discrete foci of bright fluoresence in the cell nucleus when visualised under the fluorescent microscope (Fig 7.1, see colour plate page 139) at 1 day post infection. Fluorescence was detected in the VvL1$_R$-16 infected cells reacted with antiserum to the VLP-16 specific antiserum (Fig.7.1a, anti-VLP-16 antiserum) as well as detecting group-specific antigen (Fig 7.1b, anti-BPV-1 antiserum). No fluorescence was seen in the uninfected control cells, reacted with either antibody preparation (Fig.7.1c).

7.3.2. Mouse challenge model

7.3.2.1. BALB/c mice challenge system

To determine whether VLP-16 would protect mice against challenge with VvL1$_R$-16, serial dilutions of ovarian extracts from mice challenged with VvL1$_R$-16 were titrated on CV-1 cells to obtain the difference in titre of rVv in vaccinated and unvaccinated mice ovaries. The result of the 8 BALB/c mice ovarian titiration after rVv challenge is shown in Table 7.1. Four mice were vaccinated with 10μVLP-16 in PBS plus 10% ICE. Four mice were left unvaccinated as controls. The degree of protection, $\Delta\log_{10}$, was estimated as the difference between the mean $\log_{10}$ ovarian titre of vaccinated and unvaccinated mice.

Table 7.1. Ovarian titres in female BALB/c mice challenged with $5x10^6$ pfu $VvL1_R$-16

Mouse	Pfu per mouse	Log_{10} titer	Mean log titer (± SEM#)
A.Vaccinated*	$2x10^7$	7.3	
B.Vaccinated*	$1.5x10^7$	7.2	
C.Vaccinated*	$1.7x10^7$	7.2	
D.Vaccinated*	$1.1x10^7$	7.0	7.2 (±0.06)
E.Unvaccinated	$6.5x10^{11}$	11.9	
F.Unvaccinated	$2.4x10^{11}$	11.3	
G.Unvaccinated	$1.5x10^{12}$	12.2	
H.Unvaccinated	$4.1x10^{11}$	11.6	11.8 (±0.19)

* Mice vaccinated with 10μg VLP-16 in PBS plus 10%ICE
# SEM = Standard error of the mean

The Δlog_{10} was 4.6, calculated as the difference between the mean log titre of the unvaccinated mice and the vaccinated mice. This 4.6 log_{10} was the degree of protection afforded to the vaccinated mice by VLP-16. The small difference in the ovarian titres of the individual mice in each group, was noteworthy.

7.3.2.2. C57/BL6 mouse challenge system

The C57/BL6 mice were assessed for use in the challenge model. The results are shown in Table 7.2. Here the Δlog_{10} protection was 2.3 between vaccinated and unvaccinated C57BL/6 mice, challenged with $VvL1_R$-16. A graphic comparison of the degree of protection in BALB/c and C57BL/6 is shown in Fig 7.2.

Table 7.2. The ovarian $VvL1_R$-16 titre in C57BL/6 mice after challenge

Group (3 mice)	Challenge	Pfu/mouse	Log_{10}/pfu (± SEM#)
A. vaccinated	$VvL1_R$-16	$3x10^7$	7.5(±0.04)
B unvaccinated	$VvL1_R$-16	$6.5x10^9$	9.8(±0.04)

*Mice vaccinated with 10μg VLP-16 in PBS plus 10%ICE, # SEM = Standard error of the mean

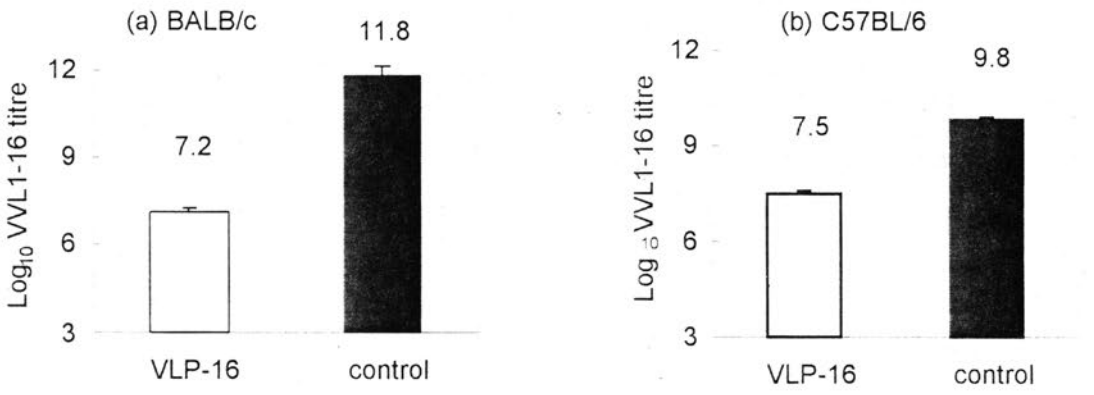

Fig. 7.2. VLP-16 immunisation protects mice against challenge with L1-16 expressed by vaccinia virus. (+) Unvaccinated control or (1) VLP-16-immunised (a) BALB/c and (b) C57BL/6 mice ovarian titres were measured 5 days after challenge with $VVL1_R$-16. Ovarian titres per mouse are expressed as the log_{10} of the number of plaque forming units. Each bar represents the mean log_{10} VVL1-16 pfu (± SD). In (a) the difference is significant $P< 0.05$ (student t test).

7.3.2.3. Vaccinia virus wild type challenge

In order to assess the effect of challenging VLP-16 vaccinated and unvaccinated mice with wild type Vv, the ovaries were titrated 5 days after challenge and the results are shown in Table 7.3.

Table 7.3. The protection afforded to BALB/c mice vaccinated with VLP-16 and challenged with wild type Vv

Vaccination	Challenge	Log_{10} ovarian titre	Protection Δlog_{10}
VLP-16	Vv wild type	7.4	1.0
No vaccination	Vv wild type	8.4	

VLP-16 vaccination was able to afford a 1.0 log_{10} protection to challenge from Vv wild type, compared with unvaccinated mice.

7.3.2.4. Assessment of protection by rBCG

It was important for the consideration and future development of rBCG constructs as candidate vaccines, to assess their relative efficacy in affording protection to BALB/c mice from challenge with $VvL1_R$-16. Mice were vaccinated with wild type BCG or recombinants pMV361-L1n rBCG, pNIV-$L1_{tr}$ rBCG and pNIV-L1 rBCG. There were

three mice per group and the ovaries from each group were combined for viral extraction and titration. The results are presented in Table 7.4.

Table 7.4. The protection afforded to BALB/c mice vaccinated with rBCG L1-16

Vaccination	Challenge	Log_{10} ovarian titre (± SEM#)	Protection Δlog_{10}
A. wild type BCG	$VvL1_R$-16	8.5 (±0.3)	3.4
B. pMV361-L1n rBCG	$VvL1_R$-16	7.3 (±0.3)	4.6
C. pNIV-$L1_{tr}$ rBCG	$VvL1_R$-16	7.4 (±0.5)	4.5
D. pNIV-L1 rBCG	$VvL1_R$-16	7.6 (±0.3)	4.3
E. pNIV-L1 rBCG	Vv wild type	8.5 (±0.6)	1.8
F. unvaccinated	Vv wild type	10.3 (±0.3)	
G. unvaccinated	$VvL1_R$-16	11.9 (±0.7)	

The best protection of 4.6 Δlog_{10} was afforded by pMV361-L1n rBCG, with similar protection afforded by the pNIV-$L1_{tr}$ rBCG and pNIV-L1 rBCG of 4.6 and 4.3 Δlog_{10} respectively. Wild-type BCG was itself able to afford a 3.4 Δlog_{10} protection against rVv challenge. The pNIV-L1 rBCG vaccinated mice challenged with wild type Vv were shown to be afford a 1.8 Δlog_{10} protection in the vaccinated mice compared with unvaccinated mice.

7.3.2.5. The assessment of an oral VLP-16 vaccine

Mice were immunised intragastrically with three different concentrations of VLP-16, three times at two weekly intervals. The mice were challenged 6 weeks after the last immunisation with rVv to assess the ability if this route of VLP immunisation to induce protection in these mice. The results of the ovarian titration of mice orally fed with VLP-16 is shown in Table 7.5. Protection against challenge was afforded to all immunised mice, but maximal protection (2.2 Δlog_{10}) was obtained for the 50μg dose.

Table 7.5. Ovarian titres of control mice and mice immunised intragastrically and challenged with $VvL1_R$-16

Vaccination	Challenge	Log_{10} ovarian titer	Protection Δlog_{10}
VLP-16 (100μg)	$VvL1_R$-16	7.3	0.8
VLP-16 (50μg)	$VvL1_R$-16	5.9	2.2
VLP-16 (10μg)	$VvL1_R$-16	7.1	1.0
No vaccination	$VvL1_R$-16	8.1	

7.3.2.6. The verification of the presence of rVv

It was important to determine that rVv was maintained after challenge in the mice and that there had been no reversion to Vv wild type. CV-1 cells infected with the ovarian extracts and overlaid with X-gal stain, showed blue CV-1 cells confirming that the rVv genotype, expressing the *Lac* Z gene, was maintained.

7.4. DISCUSSION

This chapter has described the establishment of a successful mouse challenge model for the quantitative assessment of the protective efficacy of potential HPV vaccines. VLP-16 was shown to afford a $4.6log_{10}$ protection in i.p. vaccinated mice compared with unvaccinated mice and similar protection was afforded by 3 potential rBCG vaccines. A VLP-16 vaccine administered to mice intragastrically was shown afford some measure of protection against challenge.

The use of an early promoter to express L1 from Vv was imperative to the stimulation of the CMI responses required in this study. However, the HPV-16 L1 open reading frame contains two repeats of the sequence TTTTTNT at positions 5626 to 5633 and 6375 and 6383, which may function as termination signals for the Vv DNA-dependent RNA polymerase. [Zhou *et al.*, 1991]. This would therefore result in the production of a severely truncated protein, which would not be able to form conformational structures [Sapp *et al.*, 1998]. Our results confirm some read through of the RNA polymerase and the

production of L1 as detected by immunofluorescent staining, using an antibody (079) to conformational epitope on intact VLP-16 (Fig 7.1a). Further tests (e.g. Western blot) will have to be performed to determine the length of the expressed L1 protein.

VLP-16 immunised BALB/c mice were better able to control $VvL1_R$-16 infection following challenge than unvaccinated control mice ($4.6\log_{10}$ protection, $P = 0.0001$; Fig. 7.2a). Protection was VLP-16 specific as challenge with wild type Vv only resulted in a one $\log_{10}$ reduction in vaccinated mice ovarian virus titre ($2.4x10^7$ pfu) compared to unvaccinated mice ($2.3x10^8$ pfu) (Table.7.3), indicating some non-specific protection by innate immunity induced by Vv. X-gal staining of titration plates containing virus from mice ovaries confirmed that the residual virus in vaccinated mice was recombinant $VvL1_R$-16. There was no evidence of the reduction in viral replication *in vivo* of rVv compared to wild type Vv in the mice ovaries (Table 7.4.) as reported by Buller *et al.* [1985] who described a decreased virulence of rVv with a TK^- phenotype. There has also been a noted difference in the immune response to foreign gene products expressed from TK^+ and TK^- rVv. The different phenotypes differed in their stimulation of CTL responses [Andrew *et al.*, 1989]. This should be noted in attempts to refine this challenge model. Further refinement would be a possible alternate routes of VLP and viral inoculation as this does affect immunogenicity. The i.p. viral inoculation route used in this study is reportedly best for the production of high levels of specific CTL [Andrew *et al.*, 1989]. This is particularly so when the inoculation dose is high (10^7 pfu), but not for viral doses of 10^4 pfu, when there can be no detectable CTL. In the present study, an increase in VLP dose could possibly reduce the amount of residual virus detected in immunised mice.

Protection is by a T cell response characterised by the production of both Th1 and to a lesser extent, Th2 cytokines [Marais *et al.*, 1999]. The documentation of the control of primary of Vv infection (and most viral infection) as T cell mediated was made in several classical studies [Hirsch *et al.*, 1968; Blanden, 1974], whereas antibodies contribute to complete viral clearance. Binder and Kundig [1991] confirmed that protection against primary rVv infection was $CD8^+$ mediated with possibly some $CD4^+$ T cell involvement against small challenge doses of virus but antibodies playing no demonstrable role in protection. Bachman *et al.* [1994] in a mice protection assay against rVv challenge, showed that VSV viral proteins triggered a long lasting $CD8^+$ T cell-mediated antiviral immunity, but only when associated with cellular debris which functioned as an adjuvant.

The protein alone was only able to induce a meager CTL response. They explain this phenomenon as efficient cross-priming by the proteins released from lysed cells, which are taken up by APC and presented by correct antigens. The present study was not able to confirm this, as no difference was found in the degree of protection obtained with or without insect cell extract but it was assumed that all the VLP preparations contained some insect cell proteins which co-purified with the VLPs. This will have to be clarified with refinement of the mouse model.

Challenge experiments using VLP-16 immunised C57BL/6 mice confirmed that they were also protected from $VvL1_R$-16 challenge compared with their unvaccinated littermates ($2.3\log_{10}$ protection), but the level of protection was lower compared with BALB/c mice (Fig. 7.2b). This finding is consistent with reports showing that C57BL/6 mice are genetically more resistant to poxvirus infection than the innately susceptible BALB/c strain [O'Neill & Brenan, 1987]. In the latter, the recruitment and proliferation of CTLs is delayed for 5 days compared to the 3 days of the C57BL/6 mice. The delay could account for the more rapid transmission of virus to various organs in BALB/c mice, before the cellular immune response is initiated and the resultant higher titre of virus in the ovaries. These results indicate that BALB/c mice may be a better strain for the $VvL1_R$-16 challenge model, with a resultant higher protection factor after VLP-16 vaccination.

The ability to titrate Vv in ovaries offers a quantitative measure of the protective immunity induced in mice by potential HPV prophylactic vaccines. Recombinant BCG vaccines were all shown to afford similar protection in the mouse challenge model, which was similar to that of 4.6 $\log_{10}$ afforded by the VLP-16 preparation. The two rBCGs without NLSs (pMV361-L1n rBCG and pNIV-$L1_{tr}$ rBCG), were expected to produce L1 proteins more accessible to the immune system because of their cytoplasmic location, although this has not been confirmed. The immune response and resulting degree of protection did not appear to differ significantly with the rBCGs without or without NLSs. The two rBCGs without NLSs were able to afford a slightly higher degrees of protection (4.6 and $4.5\log_{10}$, respectively), compared with pNIV-L1 rBCG, with a full length L1 and a NLS ($4.3\log_{10}$). The immunostimulant properties and the high degree of protection afforded by wild type BCG vaccination against Vv challenge has been reported [Werner, 1978]. In that study, a 92% (23/25) protection of BCG vaccinated mice was observed 30 days after Vv challenge with 10^4 pfu, compared with 8% (2/25) in control unvaccinated mice. This was also

demonstrated in the present study by a $3.4\log_{10}$ protection from $VvL1_R$-16 by wild type BCG. Equally pNIV-L1 rBCG vaccination afforded a $1.8\log_{10}$ protection to Vv wild type challenge.

The assessment of the oral VLP-16 vaccine in BALB/c mice (challenged i.p.) indicated a measure of protection that appeared dose-dependant, with an improved protection afforded by a 50μg dose over a 10μg dose. It is unclear why the 100μg dose did not provide an improved protection over the 50μg dose, but it could be inducing tolerance in the mice. The demonstration that an oral VLP vaccine can induce protection is an important finding for the development of oral HPV vaccines. It has been shown that oral vaccination with VLPs in mice induces serum antibodies that are neutralising [Rose *et al.*, 1999], which is important for a prophylactic vaccine [Murakami *et al.*, 1999]. However, the induction of a T cell response is the requirement of a therapeutic HPV vaccine [Murakami *et al.*, 1999] and for the control of existing in HPV infection. It remains to be established what the optimal oral VLP dose would be for maximal protection from VvL1-16 challenge.

Future studies are needed to examine whether there is cross protection between different HPV VLP types and the relative advantages of different routes of VLP vaccine administration and virus challenge. The timing of the challenge relative to the vaccination and the dosage used could also affect the level of protection induced and these factors should be examined in order to optimise this system. The model could be used to directly measure the immune response following mucosal immunisation and challenge, which would more closely resemble the HPV infection in humans. These results confirm others indicating that VLPs are efficient candidates for a prophylactic vaccine.

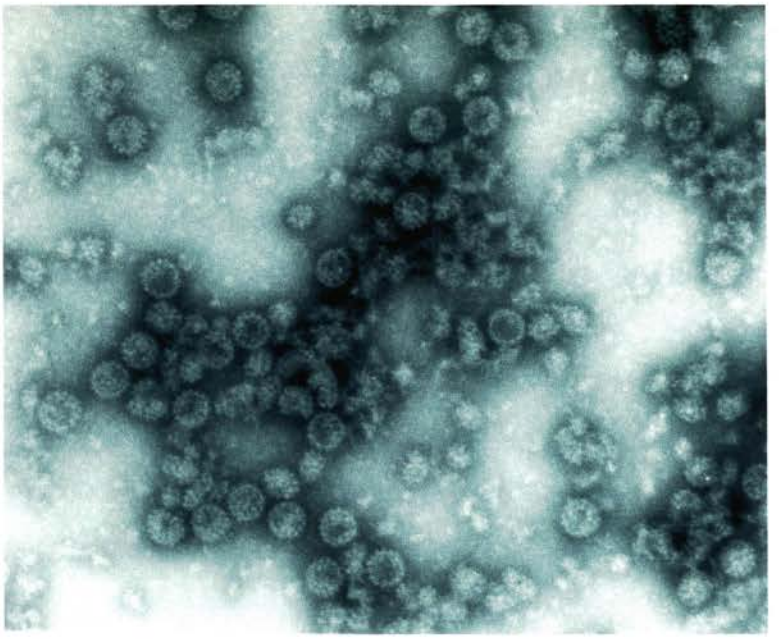

Fig. 1.7. Electronmicrograph of HPV-45 virus-like particles (supplied by Dr. R. Rose). Electron micrograph by Dr. L. Stannard.

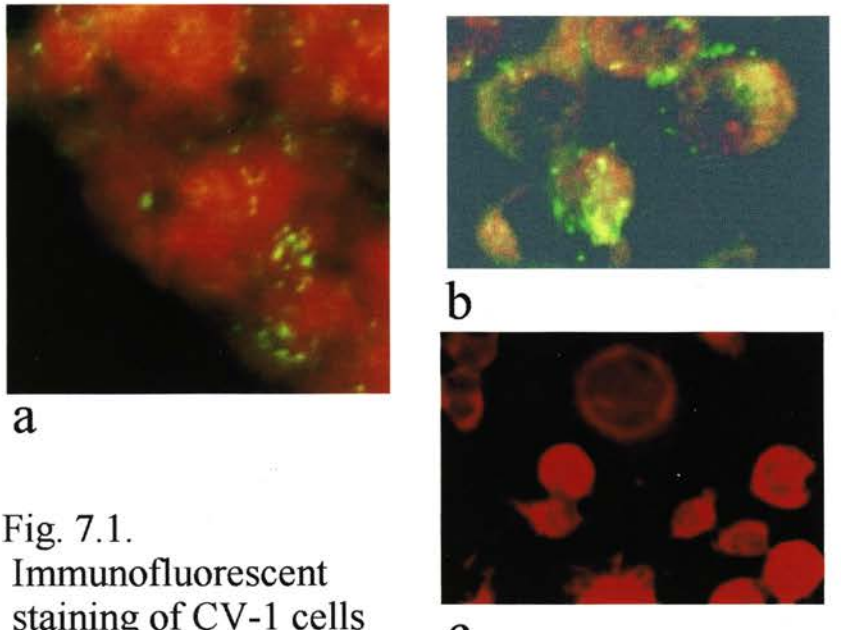

Fig. 7.1. Immunofluorescent staining of CV-1 cells infected with VvL1-16 (a) and (b), or uninfected (c). In (a) and (c) the cells were incubated with antiserum (079) to intact VLP-16 and in (b) the cells were incubated with antiserum to BPV-1.

CHAPTER 8 CONCLUSIONS

The major portion of this thesis assessed the results of assays for the presence of antibodies to HPV antigens in serum, cervical secretions and oral fluid from women with cervical disease as well as normal healthy members of the population. Serum antibodies were found to all 5 oncogenic HPV VLP types in all the population groups under study, indicating exposure to all types in the community. Seropositivity to multiple types was common, especially in older women with CaCx. Disease association was found for antibodies to VLP-16 and VLP-45 in women with CIN. There was also disease association found for antibodies to the E2-16 peptide in women with CIN. HPV and particularly HPV-16 infection as an important risk factor of invasive cervical squamous cell carcinoma [IARC/WHO, 1995] has been confirmed by its presence in women with CIN in the present studies and in those with CaCx. With the knowledge of the high prevalence of CaCx in this country and the perceived greater exposure to HPV, it was not surprising to find the relative risks of disease were no higher and sometimes lower than other parts of the world. The high seroprevalence of antibodies to VLP-45 in women with CIN and CaCx found in this study will have to be supported by the evaluation of the presence of HPV-45 DNA at the cervix of these women. HPV-45 could be a significant factor in the high prevalence of cervical disease in this country.

The study was able to show that if a woman with CIN had a confirmed HPV-16 DNA positive lesion at the cervix there would be an anti-VLP-16 antibody response of at least one iso-type in serum, cervical mucus or oral fluid. This could be a significant in the detection of the HPV types associated with disease and possibly a multi-fluid test could provide the desired confirmation of the presence of oncogenic HPV types in women with cervical disease. It remains however to be confirmed from which body fluid antibodies best reflect the HPV-associated lesion under question. Results from this study indicate that it could be an anti-VLP serum IgG response that most precisely does so. The HIV status of the women with cervical disease in this study was unknown. The study however showed an altered cervical and systemic immune response in HIV-positive women, compared with those women who are HIV-negative and with the high rate of HIV infection in South Africa, depending on region, up to 30% of women with CIN could be HIV-positive [DOH,

1999]. It will be important in the future to consider HIV status when assessing the immune responses of women with cervical disease.

The prevalence of antibodies was found to be age dependent, in that antibodies to VLP-16 were significantly more prevalent in younger women and antibodies to E2-16 in older women. Thus, the importance of the consideration of age when determining the presence of HPV antibodies in the serum of women with cervical disease was made evident. The factor of age and its part in the immune response to infection with HPV has however not been clarified. Age has been reported as a non-contributing factor to the prevalence rate of antibodies to VLP-16 by some investigators [Dillner *et al.*, 1996; Chua *et al.*, 1996; Kjellberg *et al.*, 1999]. Others [Wideroff *et al.*, 1995; Nonnenmacher *et al.*, 1996; Strickler *et al.*, 1999; Hamsikova *et al.*, 1998] have shown age to affect antibody prevalence. The present study has shown a lower prevalence rate of anti-VLP-16 IgG in older women (with cervical disease and without disease) and possibly in individuals who do not have frequent new sexual contacts, exposure to HPV would decrease and consequently HPV-specific antibodies would wane. The reason for a corresponding rise in antibodies to E2 with age in women with CIN, which was shown in this study, is unclear. Age and its relation to antibodies to HPV infection requires further assessment.

One of the most striking factors to emerge from assays using peptide ELISAs in this study, was the large number of children who had antibodies to oncogenic HPV types. The 44.5% seroprevalence found in children to the E2 peptide (245-16) of HPV-16 (E2-16) was shown to be age dependent, being highest at age 3 years (55%) and decreasing to 20% at age 12 years. The seroprevalence of antibodies to the 5 VLP types was also highest in children aged 3 years., If the antibodies are HPV type specific this indicated early acquisition of the HPV infection, with high antibody prevalence and the antibody prevalence rates decreasing in older children. The presence of antibodies to VLP-16, -18, -31, -33 and -45 in children which were detected in the present study was confirmed by others for VLP-16 [Lund *et al.*, 1997; Cubie 1998] and VLP-16, -18 and -33 [Hamsikova *et al.*, 1998; af Geijersstam *et al.*, 1999]. These reported seroprevalence rates in children were lower however, than those found in the present study and there could be a number of reasons for this difference. This country has one of the highest CaCx rates in the world and this study has found evidence of high seroprevalence to a number of HPV types, both in normal women and women with cervical disease. With so many women infected with genital HPV types, transmission to

infants by their mothers would be high and there now is convincing evidence that this occurs [Cason *et al.*, 1998; Rice *et al.*, 1999].

To compound this, HIV-positivity has been determined as being higher than 30% amongst women at antenatal clinics in certain parts of South Africa [DOH, 1999]. The present study found a 85% HPV DNA prevalence in HIV-positive women and these women could transmit HPV to a large percentage of their children. Transmission of HPV from mother to infant may occur, but sexual abuse must also be considered as a factor incurring the presence of antibodies to genital HPV types in older children. There is evidence of sexual abuse in many children under 15 years in South Africa and this could be one of the reasons the present study found serum antibodies to at least one oncogenic HPV VLP type in >20% of children. The development of HPV vaccines makes it imperative to understand the natural history of HPV infection in children. Not only will it be important to assess when to administer an HPV vaccine but the early infection by HPV-16 or other types, might have implications for the child for the acquisition or not of cervical disease later in life. There is a possibility that protection could be induced by early HPV infection, or it will be important to determine whether an infected child will have a persistent infection and face the possibility of a latent infection being reactivated later in life. This information will require longitudinal studies over many years, following children into adulthood. The oral test for HPV antibodies, which has been shown here to effectively detect anti-VLP-16 antibodies could assist in the undertaking of such a study.

Little research has concentrated on looking at the immune response to multiple HPV types and their joint effect on the risk of cervical disease. The present study found that the prevalence of anti-VLP IgG antibodies to multiple HPV types in both normal women and those with cervical disease was common, and also prevalent in children. Different prevalence rates to the 5 VLP types were found in different population groups, but all groups studied displayed antibodies to the 5 oncogenic types. This study found too that the presence of anti-VLP IgG antibodies to multiple types was especially common those with CaCx and most common in older women with CaCx. Loustarinen *et al.* [1999], looking at the multiplicative effects of infection with HPV-11/6, -16, -18,and –33, found that instead of the expected multiplicative odds ratios of the joint infections, there was antagonism in the reaction between HPV-11/6 and HPV-16 and HPV-16 and –18 and HPV-16 and -33. That is, a less than expected risk among women seropositive to both HPV-16 and HPV-

11/6 for CaCx. This was interpreted as in line with the theory of competition between serotypes of a pathogen [Lipsitch, 1997] and that there may be a cross-protective cell-mediated immunity induced between types. This would explain their finding that there was not an increased risk of CaCx with infection by multiple types. This would indicate that in the present study the women with antibodies to multiple VLP types were at no more risk of CaCx than were women seropositive to only one type. This would also be concordant with the theory of broadening of the antibody response with time to include new epitopes discussed in previous Chapters [Wang *et al.*, 1996]. The women with CaCx showing seropositivity to multiple HPV types in the present study, could have displayed antibodies to recent HPV epitopes that could be more cross-reactive and hence cross protective. This also supports the concept that vaccination against HPV might control HPV infection and that possibly a vaccine would not have to include all HPV types.

The highly complex nature of the humoral immune response to HPV VLPs and other antigens has been evidenced in the preceding chapters, with a high seroprevalence of antibodies to certain HPV-16 antigens in some women but not in others with the same grade of cervical lesion. There could be a number of reasons for the different antibody responses. (1) There have been differences in serum HPV antibody prevalence rates determined with regards to age and to disease stage [Vonka *et al.*, 1999]. In the present study, antibodies to E2-16 were prevalent in the majority of women with CIN, and antibodies to the E7 protein of HPV-16 were hardly detectable in women with CIN, but were found in the majority of women with CaCx. This could relate to the decreased expression of the E2 protein after viral integration and the concomitant increase in the expression of the E7 protein. (2) Antibodies have also been shown to persist [af Gieijersstam et al., 1998; Carter *et al.*, 1996] in normal women without cervical disease, despite the fact that about 70% of infections in women without disease are cleared [Hildesheim *et al.*, 1994; Evander *et al.*, 1995]. So seroprevalences to HPV VLPs are probably best described as markers of lifetime cumulative HPV exposure [Olsen *et al.*, 1997; Kjellberg *et al.*, 1999]. (3) Carter *et al.*, [1996] described the lack of correlation between the presence of HPV DNA and antibodies to VLP-16, since antibodies tend to develop well after HPV DNA is detected and persist after the DNA is no longer detected. (4) HPV-16 produces low yields of virus particles in cervical lesions compared to HPV-11 and HPV-6, which could be intrinsic to HPV-16 or a consequence of its sequence [Pushko *et al.*, 1994]. This low level of virion production could induce low levels of anti-HPV-16

antibodies. HPV-16 could be less immunogenic and consequently the immune response to HPV-16 could be reduced. Infection in some could have produced low titres of antibodies which were undetectable by the assay methods used, and could explain why in the present study, antibodies in some fluids of HPV-16 infected individuals were not detected. These factors could all contribute to why women studied at one time point, as in the studies discussed here, will have antibody responses that do not correspond with those of other women with the same cervical condition. Longitudinal studies over time with the correlation of multiple sero- and other reactivities (cervical and oral) over a long period, relating them to the course of disease, could be more useful in determining the correlates of disease and those of a successful immune response to HPV infection.

Bontekes *et al.* [1999] in such a multi-response study, have concluded that it is the presence of systemic IgA to VLP-16 which determines clearance of infection and systemic IgG which indicates persistence of disease. Bontekes *et al.* [1999] concluded too that local cervical responses do not predict clearance of disease and are probably more effective at onset of infection in eliminating virus. The present study supported the theory of Bontkes *et al.* [1999] in finding that it was the anti-VLP-16 serum IgA response in the HIV-negative sex-workers that appeared to be relevant to their ability to cope with their excessive HPV exposure. The antibody response at the cervix of HIV-positive women was shown to be enhanced, compared to HIV-negative women and did not reflect viral clearance. There have been no reports on the presence of HPV antibodies in HIV-positive women and this would be an important area to pursue. The further examination of immunosuppressed women, such as those with HIV infection, studying their immune responses both systemically and locally could possibly help elucidate the nature of the immune response in normal women which enables them to cope with HPV exposure or clear HPV infection.

A most important direction for further research must be to examine the immune response at the cervix, the site of entry of the virus. The present study was not able to confirm how the presence of cervical antibodies related to cervical disease. It was able to show that more women with CIN had an anti-VLP-16 IgA antibody response at the cervix than did women without disease. The study also showed that fewer HIV-negative women have a cervical anti-VLP-16 IgA or IgG responses compared with HIV-positive women. However before firm conclusion about cervical IgA in women with CIN can be made, it will be important to relate the presence of antibodies to HPV antigens at the cervix with the stage of the

menstrual cycle. The effects of hormonal and other factors such as pregnancy on cervical mucus antibody responses, have been demonstrated [`Kutteh *et al.*, 1998] and these should be considered in future work.

Much attention has focussed on the nature of the immune response at other mucosal surfaces such as the gut and bronchial areas [Ogra *et al.*, 1999] but the immune response in the female genital tract requires more clarification. Most viruses stimulate IgA at local areas where they infect [Feldman, 1993]. This secretory IgA may protect the epithelial surface and be most important in the initial stages of infection for a virus like HPV, which does not have a viraemic phase. Recent reports indicate that a CTL response is also important for resistance to mucosal viral transmission. Belyakov et al., [1998] showed that long-lasting immune resistance to HIV at the murine mucosa is accomplished by $CD8^+$ CTL, and that this resistance was enhanced by the local administration of IL-12. The tracking of immune cells from cervical mucosal lesions will be important too in the understanding of the link between the cervical mucosal immune response and the systemic immune system and the part the genital mucosal system plays in the CMIS. The presence of anti-VLP-16 IgA in oral secretions as demonstrated in the present study, would support inclusion of the female genital tract in the CIMS as discussed in Chapter 5.

The mouse challenge model of the present study has shown that a CTL response was successful in conferring protection to challenge with recombinant vaccinia virus expressed HPV-16 L1 in mice vaccinated with VLP-16 both systemically and orally. The potential of VLP vaccines was demonstrated and supports other VLP vaccine research. Importantly the mouse challenge model was able to demonstrate that not only was an oral VLP-16 vaccine feasible but orally applied VLP-16 was also able to elicit a protective T cell response. . The model has shown the efficacy of potential rBCG vaccines in the protection against viral challenge. Future work with the mouse challenge model will involve the confirmation of cross protection between HPV VLP types, an important consideration in future vaccine studies.

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APPENDIX 1

Single and three letter amino acid designations

Amino acid	Abbreviation 3-letter	Abbreviation 1-letter
Alanine	ala	A
Arginine	arg	R
Asparagine	asn	N
Aspartic acid	asp	D
Cysteine	cys	C
Glutamic acid	glu	E
Glutamine	gln	Q
Glycine	gly	G
Histidine	his	H
Isoleucine	ile	I
Leucine	leu	L
Lysine	lys	K
Methionine	met	M
Phenylalanine	phe	F
Proline	pro	P
Serine	ser	S
Threonine	thr	T
Tryptophan	trp	W
Tyrosine	try	Y
Valine	val	V

APPENDIX 2

Phosphate buffered saline (PBS) pH 7.4

For 1 litre.

Sodium Chloride	NaCl	8 gm
Potassium dihydrogen orthophosphate	$KH_2 PO_4$	0.2gm
Disodium hydrogen orthophosphate 12-hydrate	$Na H PO_4 12H_2 0$	2.9gm
Potassium Chloride	KCl	0.2gm

Sodium carbonate buffer (10mM) pH 9.6

For 1 litre

Sodium carbonate	Na_2CO_3	1.59gm
Sodium hydrogen carbonate	$Na HCO_3$	2.93gm
Sodium Azide	NaN_3	0.2gm