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Title Characterisation of Immune Responses to the E5 Protein of the Human Papillomavirus Type 16

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CHARACTERISATION OF IMMUNE RESPONSES TO THE E5 PROTEIN OF THE HUMAN PAPILLOMAVIRUS TYPE-16

by

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A thesis submitted to the University of Luton for the degree of Doctor of Philosophy

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ABSTRACT

High-risk mucosal human papillomaviruses (HPVs) are major aetiological agents for the development of cervical cancer. Thus, the current goal of cervical cancer treatment is to develop vaccines against HPVs. Such vaccines would either prevent cervical cancer by eliminating HPV infection or be useful for treating established lesions by the destruction of cells displaying HPV proteins.

The aim of this thesis was to characterise immune responses to the E5 protein of HPV-16, one of several antigens with possible use in vaccination. To determine whether immune responses to HPV-16 E5 existed and whether they could be correlated with disease severity or with the presence of HPV-16 DNA, both cell mediated (Chapter Two) and humoral (Chapter Three) immunity was investigated in women with and without cervical disease. Cellular responses in a minority of women were inversely correlated with disease severity. However, E5 specific antibodies were negatively correlated with the absence of HPV-16 DNA. Thus, although some immune responses were evident, these were generally limited to a small number of subjects and were not associated with the detection of HPV-16 E5 mRNA or DNA sequence variants.

Due to the immune responses in women, E5 was further investigated to determine if the absence of HPV-16 E5 specific immune responses was due to the poor antigenicity of HPV-16. Mice were immunised with synthetic peptides corresponding to full length HPV-16 E5 (Chapter Four). As with the human data, cellular responses and weak antibody responses were detected in mice. Some mice also exhibited cytotoxic T-lymphocyte responses and when E5/major histocompatibility class I (MHC-I) interactions were investigated, a number of peptides showed a high percentage of binding.

The E5/MHC-I interactions were further investigated (Chapter Five). The surface expression of MHC-I on cells containing HPV-16 or -18 DNA was found to be lower than on HPV DNA negative cell lines even after stimulation with interferon-gamma. Stimulation with E5 synthetic peptides increased expression of cell surface MHC-I molecules on cell lines negative for HPV DNA. Furthermore, the presence of the E5 gene reduced the expression of the ovalbumin gene in normal human keratinocytes.

In conclusion, the data contained within this thesis indicate that HPV-16 E5 CMI is inversely correlated with disease status. It is possible to induce cell mediated responses to HPV-16 E5 and low-titre antibody responses. The presence of HPV-16 E5 DNA may impair normal cellular function.

This thesis is dedicated to the loving memory of my father

Bhajan Singh Gill

1941-1997

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Abbreviations

The abbreviations in this thesis are listed below, in some instances identical abbreviations have multiple definitions but the appropriate choice of definition is clear in the text.

α	alpha
A	Adenine
A	alanine
AP-1	activator protein-1
A_{XXX}	absorbance at XXX nanometers
BCG	Bacille Calmette Guerian
bp	base pair
BPV	bovine papillomavirus
BSA	bovine serum albumin
C	cysteine
C	cytosine
CA	condyloma accuminata
CD	cluster of differentiation
CgPV	Columbus guereza papillomavirus
CMI	cell mediated immunity
CO_2	carbon dioxide
COOH	carboxylic terminus
COPV	canine oral papillomavirus
CRPV	cotton-tail rabbit papillomavirus
CTL	cytotoxic T-lymphocyte
D	aspartic acid
D	downstream
DMEM	Dulbeccos modified Eagles media
DMF	dimethyl formamide
DNA	deoxyribonucleic acid
dNTP(s)	deoxynucleoside tri phosphates
DPBS	Dulbeccos modified phosphate buffered saline
DTH	delayed type hypersensitivity
E	early
ECACC	European collection of cell cultures
E.Coli	*Escherichia coli*
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EGF-R	epidermal growth factor-receptor
EIA	enzyme immunoassay
EmRNA	early messenger ribonucleic acid
EV	*Epidermodysplasia Verruciformis*
FBS	foetal bovine serum
Fet	Fishers' exact test
Fmoc	9-fluorenylmethyloxycarbonyl

G	guanine
GM-CSF	granulocyte-macrophage colony stimulating factor
h	hour
H	histidine
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
H_2O	water
HOBt	1-hydroxybenzotriazole
HPV	human papillomavirus
HRP	horse radish peroxidase
HSV	herpes simplex virus
H-SIL	high grade squamous intraepithelial lesion
I	isoleucine
I.A.R.C.	International Agency for Research on Cancer
IgA	immunoglobulin class A
IgG	immunoglobulin class G
IgM	immunoglobulin class M
IL-2	interleukin-2
IL-4	interleukin-4
IL-6	interleukin-6
IFN-α	interferon-alpha
INF-γ	interferon-gamma
IPTG	isopropyl β-D-thiogalactopyranoside
ISCOMs	immunostimulatory complexes
KCl	potassium chloride
L	late
L	leucine
LB	Lauria Bertoni
L-SIL	low grade squamous intraepithelial lesion
M	methionine
min	minute
mRNA	messenger ribonucleic acid
$MgCl_2$	magnesium chloride
MHC-I/II	major histocompatibilty class I/II
$M\text{-}H_x^2$	Mantel Haenszel test
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
n	number
N	asparagine
NC	normal cytology
N.C.I.	National Cancer Institute
NCR	non-coding region
NF-1	nuclear factor-1
NH_3	amino terminus
NHKC	normal human keratinocytes
N.H.S.	National Health Service
NK	natural killer
nt	not tested

Oct-1	octamer binding factor-1
OPD	o-phenylenediamine dihydrochloride
O.P.C.S.	Office of Population Census and Survey
ORF(s)	open reading frame(s)
P	proline
p53	protein encoded by the p53 tumour suppresser gene
Pap test	Papanicoloau test
PBMC(s)	peripheral blood mononuclear cell(s)
PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween
PcPV	pygmy chimpanzee papillomavirus
PCR	polymerase chain reaction
PEF-1	papillomavirus enhancer binding factor-1
pRB	protein encoded by the RB tumour suppresser gene
pt	paired T-test
PV	papillomavirus
Q	glutamine
R	arginine
Ref	reference
RFLP	restriction fragment length polymorphism
RhPV	Rhesus Monkey papillomavirus
RNA	ribonucleic acid
RPMI 1640	Rockwell Park Memorial Institute media 1640
RT-PCR	reverse transcription polymerase chain reaction
s	seconds
S	serine
SEM	standard error of the mean
SFM	serum free media
SI	stimulation index
SIL	squamous intraepithelial lesion
SPPS	solid phase peptide synthesis
SV40	simian vacuolating virus 40
T	threonine
T	thymine
TAP	transporter associated with antigen presentation
T.*aq*	*Thermus aquaticus*
TBE	TRIS borate ethylenediamine tetra-acetic acid buffer
TEF-1/2	transcriptional enhancer factor-1/2
TFA	trifluoroacetic acid
TGF-β	transforming growth factor-beta
Th	T-helper lymphocyte
T.li	*Thermus litoralis*
TNF-α	tumour necrosis factor-alpha
TRIS	(hydroxymethyl) aminomethane
U	upstream
U	units
U.K.	United Kingdom
U.S.A.	United States of America

ut	unpaired T-test
V	valine
VLP	virus like particle
v/v	volume for volume
W	tryptophan
W.H.O	World Health Organisation
w/v	weight for volume
X-Gal	5-bromo-4chloro 3-indoyl-β-D-galactopyranoside
X-mer	X amino acid long peptide
Y	tyrosine
YY-1	yin yang-1

Declaration

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of Luton. It has not been submitted before for any degree or examination in any other University.

Dilbinder Kaur Gill

21st day of December, 1999.

Chapter One

General Introduction

1.1 INTRODUCTION

Cervical cancer is the most common female malignancy in the developing world where an estimated 80% of the 500,000 new cases occur annually (Munoz & Xavier-Bosch, 1997). Worldwide it is the second leading cause of cancer related deaths among women (Parkin *et al.*, 1999). The most recent census reported 1,369 deaths due to cervical cancer in England and Wales, most of these occurred where women had not been screened for cervical abnormalities (World Health Organisation [W.H.O.], 1998). There has however, been a 24% decline in deaths due to cervical cancer since the introduction of large-scale screening in England and Wales (Office of population census and survey; O.P.C.S., 1990). In comparison, of the 8,000,000 women screened annually in the United States of America (U.S.A.), there are some 13,000 new cases of cervical cancer resulting in about 4,500 deaths annually (Boring *et al.*, 1991). When comparing death rates for the number of women screened, the U.S.A. has double the mortality (0.06%) of England and Wales (0.03%). This may reflect the benefits of a National Health Service (N.H.S.), where screening is freely available as opposed to the health insurance scheme operated in the U.S.A. Americans with the lowest standards of living have a two fold incidence of cervical cancer compared to Americans with a higher standard of living (Baquet *et al.*, 1991; Pappas *et al.*, 1993).

The current treatment for cervical cancer is minor surgery if detection occurs before invasion of the subepithelial tissue. Cervical lesions are treated using cryotherapy, surgical loop excision, laser ablation, interferons or a variety of chemical regimes but no single treatment is completely effective and recurrences are common.

In addition to ineffective treatment, the cost of surgery on a day-care basis has been estimated around about £500 per out patient and this rises to approximately £1,000 per in patient (London teaching hospitals, Jones, 1995). Of the 3.7 million women screened each year an estimated 206,200 abnormal smears are reported every year in England and Wales (Department of Health, 1997). The cost of surgery may be estimated to lie within the range of £125-250 million/year in the U.K. in addition to the £100 million spent on screening (Clear Communications, 1996).

There is strong epidemiological and molecular biological evidence suggesting that the aetiology of cervical cancer is associated with genital infection by oncogenic human papillomaviruses (HPVs). The association of an infectious agent with cervical cancer provides a unique opportunity to target this disease by vaccination. The introduction of an effective vaccine against cancer associated HPVs would prove to be a highly cost effective public health policy. In addition to cost, a vaccine would relieve the emotional distress caused to patients with abnormal smear results (Richardson *et al.*, 1996).

A prophylactic vaccine would supersede the need for population based screening programs and a therapeutic vaccine would provide treatment for pre-malignant and malignant cervical lesions. Such vaccines would benefit all women, preventing and/or treating cervical lesions. Vaccines could also be modified to prevent and treat other HPV associated disease including *condyloma accuminata* (CA), laryngeal papillomas and *epidermodysplasia verruciformis* (EV, see section 1.3.4).

The cost of mass inoculation can be estimated at around £17 per person and hence if the whole U.K. female population (27.5×10^6) were vaccinated the cost would be in the order of £467 million. Alternatively, assuming that there are approximately $0.5 \times$

10^6 women in each year group and all women were vaccinated once at prepubescent the cost would be around £8.5 million/year, resulting in huge financial savings for the N.H.S.

1.1.1 Pathological changes associated with cervical cancer

Cervical cancer occurs at the transformation zone of the uterine cervix, where cells undergo rapid turnover. These cells appear to be particularly susceptible to carcinogens, as the incidence of cancer of the cervix is greater than cancers of other female genital sites (Singer, 1982). The uterine cervix comprises the ectocervix and the endocervix. The former is covered with squamous epithelium and the latter is lined by a single layer of mucinous columnar epithelium (Figure 1.1). The area where the two cell types meet is referred to as the squamocolumnar junction. In most young women the columnar epithelium extends from the endocervix onto the ectocervix, giving rise to an area termed the endocervical ectropion. As women age, the ectocervical columnar cells are increasingly replaced by squamous epithelial cells in a process known as squamous metaplasia, the effect of which is the translocation of the squamocolumnar junction from the ectocervix to the internal Os, within the endocervix. The area between the original and the new squamocolumnar junction is referred to as the transformation zone.

The pathology of cervical cancer consists of a range of progressive histological changes (Richart & Barron, 1969). Pre-invasive changes are termed squamous intraepithelial lesions (SIL; National Cancer Institute, N.C.I., 1988) and are classified histologically to denote severity (Figure 1.2).

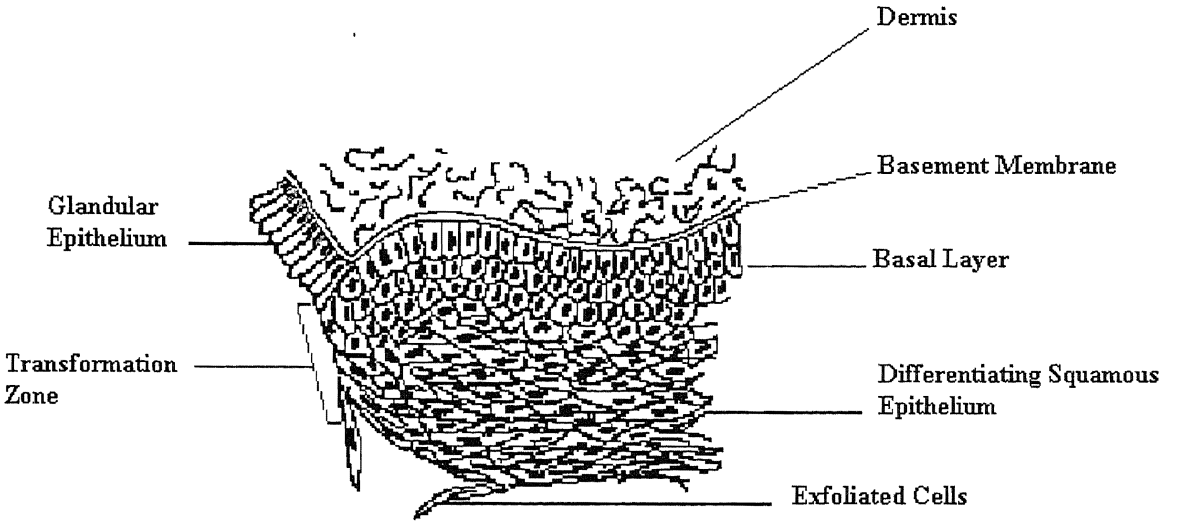


Figure 1.1 Cross sectional view through the normal cervix showing the transformation zone. The chances of detecting SIL are diminished when the transformation zone withdraws into the endocervical cancal or when a small lesion is missed. Modified from Singer, (1982).

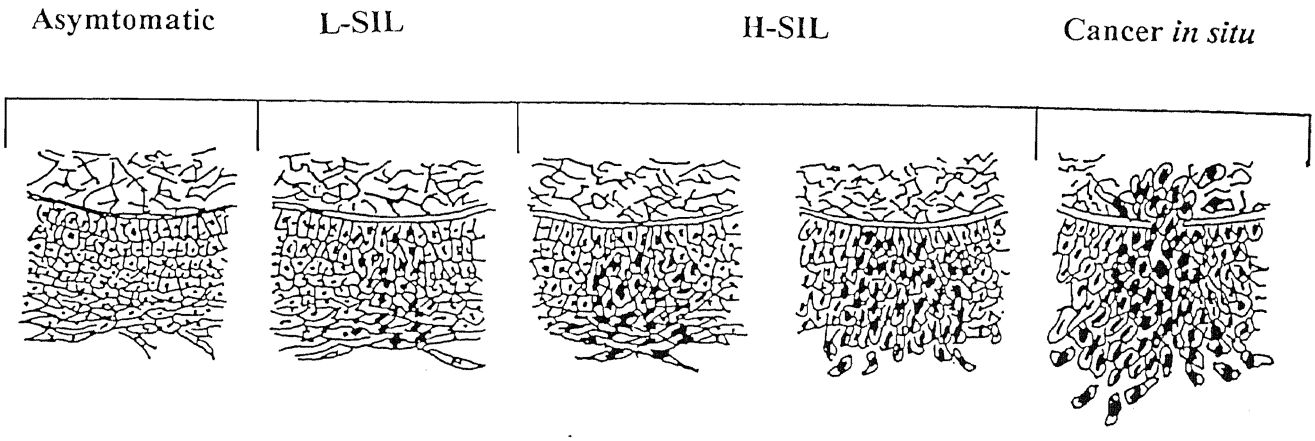


Figure 1.2 The multistep progression of cervical cancer. A negative smear result may show some normal inflammatory changes or metaplasia, SIL is sub-divided into I, II or III depending on the severity of lesion. Cervical cancer is diagnosed once abnormal cells have breached the basement membrane. The median time for the progression of L-SIL to borderline H-SIL is approximately 5 years and progression of L-SIL to H-SIL takes about 7 years (Barron & Richart, 1971). In contrast, 62% of L-SIL and 52% of H-SIL regress to normal while 30% H-SIL and 16% L-SIL progressed to cancer (Nasiell *et al.*, 1986). Modified from Richart, (1976).

Low grade SIL (L-SIL) show mild dysplasia, cells usually have a diploid or polyploid DNA content and display the cytopathic effects of HPV infection (koilocytosis). High grade SIL (H-SIL) show moderate to severe dysplasia consisting of proliferative basal type cells with a high nuclear to cytoplasmic ratio which are usually aneuploid

and monoclonal in nature. Neoplastic changes that are most often associated with cervical cancer are koilocytosis (characteristic vacoulated cells) dyskeratosis, basal cell hyperplasia and abnormal mitotic figures (aneuploidy/polyploidy; Richart, 1987).

1.1.2 The cervical smear test

The cervical smear or the Papanicoloau test (Papanicoloau, 1955) involves examination of exfoliated cervical cells to detect the abnormalities. It is based on the fact that nuclear abnormalities of SIL are present throughout the whole thickness of the epithelium and can thus be detected in cells from the surface of the lesion. A spatula is inserted into the cervical Os and rotated through 360° to harvest cells from the transformation zone (Ayer, 1949). Cervical mucus and cells adhering to the spatula are spread evenly across a glass slide, fixed in 95% ethanol in water (v/v) and stained with a trichrome mixture specifically developed for the investigation of cervical and vaginal cells (Papanicoloau, 1955).

The chances of detecting underlying SIL are enhanced by the fact that there is poor cellular adhesion between neoplastic cells and these are readily removed during the scraping process. Smears are classified as Pap (from Papanicoloau) I and II, which correspond to no significant morphological changes; Pap IIIa with mild to moderate dysplasia; Pap IIIb with severe dysplasia; Pap IV with cancer *in situ*; Pap V with invasive cancer. Women with abnormal Pap smears (IIIa, IIIb, IV or V) are referred for colposcopy and a biopsy may be taken for histological analysis to give a conclusive diagnosis of the cervical lesion.

It is estimated that an average 5-30% of cervical abnormalities are missed, but because SIL progresses slowly, these lesions may be detected at a second

examination. The false negative rate for a cervical smear has been estimated to be about 10-20% with sampling errors accounting for the greatest number of misses. For example, pathologists tested on pre-screened smears, gave a false negative error rate of approximately 5% (Davey *et al.*, 1993; Koss, 1993). There have also been isolated incidences of poor quality control within laboratories which have led to a number of preventable deaths. Even so, an estimated 3,900 cervical cancers are thought to be prevented each year in England and Wales. The cervical screening program may soon be improved by the introduction of automated screening as recommended by the W.H.O. in 1997.

Cervical screening in England and Wales offers a three or five-yearly test to women aged 25 to 64 years. Potentially cervical cancer is avoidable through effective screening and early treatment of SIL. In practice however, despite a U.K. budget for promoting the cervical screening program of £100 million/year, many women at risk of developing cancer do not attend (Hakama *et al.*, 1989; Clear Communications, 1996). Reasons for this include ignorance, embarrassment or simply that many do not perceive they are at risk. Awareness of the value of screening is still largely restricted to women belonging to those social classes least likely to develop cervical cancer (O.P.C.S., .1990).

A relationship between social status and the risk of cervical cancer exists historically, for example, during the years 1851 to 1971 the incidence and mortality from cervical cancer in England and Wales was twice as common in manual as in non-manual social classes, which long antedates cervical screening (Logon, 1982). Conversely, an improved social status, the education of women, later marriages and fewer

pregnancies resulted in a reduction in the incidence and mortality of cervical cancer in developed countries (Laara *et al.*, 1987).

In countries that have introduced cervical screening tests which employ the cytology based Pap test, death rates and incidences of cervical cancer have fallen (W.H.O., 1998). However, the number of positive smear results have been increasing, with 19% developing into cancer within 2 years in the 1970s and, 30-40% progressing to cancer in the 1990s (Kinlen & Spriggs, 1978; Buckley, 1994). This may be due to changes in sexual practices following introduction of the contraceptive pill or may represent better diagnosis with more women, especially those at risk, attending for cervical screening tests.

1.1.3 Historical infectious aetiological agents for cervical cancer

Many reviews cite Rigoni-Stern, who, in 1842, published an article that implicated a sexually transmitted factor for cervical cancer and SIL, as lesions were frequently detected in married women but were rare in nuns. Much later, investigators found cervical cancer and SIL prevalent in women with a history of multiple sexual partners, early age of first coitus (Rotkin, 1973) and previous histories of venereal disease (Beral, 1974; Singer *et al.*, 1982).

Due to the sexually transmitted nature of cervical disease, a number of micro-organisms have been investigated as a possible cause. These include, Herpes simplex virus-2 (HSV-2), *Trichomona vaginalis*, cytomegalovirus, *Neisseria gonorrhoea*, *Treponema pallidum*, protozoa and *Chlamydia trachomatis.* However, many of these associations proved to be casual rather than causal (Naib *et al.*, 1966; zur Hausen *et al.*, 1974). The low incidence of cervical cancer amongst the Jewish community was

attributed to the absence of 'oncogenic' smegma under the prepuce of the penis but this is more likely due to circumcision and the reduction of HPV harbouring tissue (Singer, 1982; see 1.3.1).

1.1.4 Evidence supporting association of human papillomaviruses with cervical disease

HPVs were first investigated as potential causative agents of cervical cancer after anecdotal reports of the malignant conversion of warts. Other factors implicating HPVs in the aetiology of human anogenital cancer include:

(i) HPV DNA is detected in at least 80% of patients with cervical cancer and progression of cervical lesions to cancer is more likely when they contain HPV-16 or -18, than HPV-6 or -11 (Durst *et al.*, 1983; Syrjanen *et al.*, 1985 Lorincz *et al.*, 1987).

(ii) Cell lines derived from patients with cervical cancer contain either HPV-16 or -18 DNA sequences integrated into host DNA, *i.e.* SiHa and CaSki cells contain HPV-16 DNA sequences and the C4-1, HeLa and SW756 cell lines contain HPV-18 sequences (Boshart *et al.*, 1984; Yee *et al.*, 1985; Schwarz *et al.*, 1985).

(iii) The presence of biological analogues. Cottontail rabbit papillomavirus (CRPV) is associated with malignant papillomas in rabbits and bovine papillomavirus type-4 (BPV-4) is associated with malignant conversion in highland cows (Shope *et al.*, 1933; Jarrett *et al.*, 1978).

(iv) *Epidermodysplasia verruciformis* (EV) is a rare inherited condition in which specific HPV types have been identified in squamous cell carcinoma developing 20-30 years after the appearance of typical wart-like lesions.: HPV types -5 and -8 but also -9, -12, -14, 15, -17, -19, -20, -21, -22, -23, -24, -25, -36, -37 and -38 have been

detected in EV (Pfister & Fuchs, 1987). In immunocompetent patients a high frequency of specific high risk HPV types have been found in cervical carcinomas and periungual squamous cell carcinoma. There is also accumulating evidence suggesting that squamous cell carcinoma occurring at other non-genital mucous membranes may be HPV related (Dehmezian *et al.*, 1987; McDonnell *et al.*, 1989; Bradshaw *et al.*, 1992).

It is now established that HPV is the central aetiological factor of cervical cancer and SIL (International Agency for Research on Cancer, I.A.R.C., 1995). Using polymerase chain reaction (PCR) assays HPV DNA is regularly demonstrated in invasive cancers from 72-100% of cancer positive samples (Gissmann, 1984; Schwarzt *et al.*, 1985; zur Hausen, 1989; Resnick *et al.*, 1990; Riou *et al.*, 1990; van den Brule *et al.*, 1991; Bergeron *et al.*, 1992; Das *et al.*, 1992; ter Meulen *et al.*, 1992). HPV types -16 and -18 predominate in invasive cancers with HPV-16 being the most frequently detected type (Durst *et al.*, 1983; Kulski *et al.*, 1987; Fuchs *et al.*, 1988). In inner south-east London, where research for this thesis was performed, approximately 62% of invasive cancers are positive for HPV-16 DNA (Cavuslu *et al.*, 1996).

1.2 PAPILLOMAVIRUSES

Papillomaviruses (PVs), from the Latin *papilla*, 'nipple or pustule' and the Greek suffix *-oma* 'tumour' are members of the Papovaviridae family (Murphy & Kingsbury, 1990). Polyomaviruses, which include Simian Vacuolating viruses (SV40), JC polyomavirus and BK polyomavirus form the second half of the Papovaviridae sub-family. These are widespread in humans and animals causing

persistent, but silent, infections of the urinary tract, and are oncogenic when injected into newborn animals. This classification is based on the common capsid structure and similarities in biochemical composition of the two groups, although there is no similarity in genomic organisation.

1.2.1 Human papillomavirus structure

Virus particles are proteinaceous, non-enveloped with a diameter of 55-60 nanometers (nm) and have an icosahedral structure of 72 capsomeres (Figure 1.3). The viral capsid is encoded by the L1 gene and consists of 72 capsomeres consisting of 60 hexomeric structures (adjacent to six capsomers) and 12 pentomeric structures (adjacent to five capsomers) arranged in a skew T=7 icosahedral surface lattice (Finch & Klug, 1965; Klug & Finch, 1965; Baker *et al.*, 1991).

Cryoelectron microscopy has shown that capsomers consist of a trunk that broadens both distally and proximally. The capsomers project to a height of 5.8 nm ending in a five pointed star shaped peak and meet at the base to form a 2 nm thick shell. A cylindrical channel of approximately 2.8 nm extends centrally within each capsomer along its axis half way to the base (Baker *et al.*, 1991). Since capsomeres display a five fold symmetry they are probably pentamers composed of five molecules of the major capsid protein L1, which contributes to 80-90% of the protein content of the shell (Baker *et al.*, 1991; Favre *et al.*, 1975; Doorbar and Gallimore, 1987).

Each virion contains a single copy of circular double-stranded DNA of 7,900 basepairs (bp) in the form of chromatin like complex with cellular histones (Seedorf *et al.*, 1985; Pfister & Fuchs, 1987; Figure 1.4). All protein-encoding sequences are located on one DNA strand and the reading frames are designated early (E) or late (L)

to indicate their expression in the viral life cycle. The single coding strand of HPV-16 DNA contains 6 E open reading frames (ORFs) and 2 L ORFs. The early region makes up approximately 45% of the viral genome, the late region makes up 40%. The rest is a non-coding region containing viral regulatory elements (NCR).

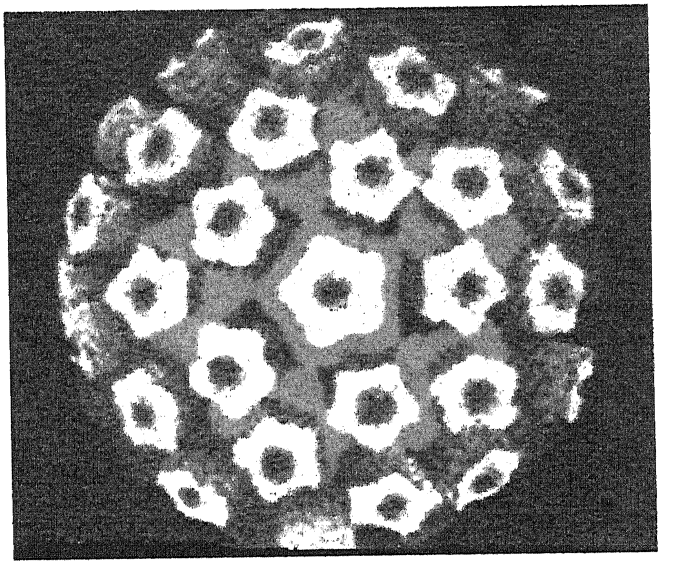


Figure 1.3 HPV capsid structure. A computer enhanced electron micrograph of HPV-1. Capsomeres display a five-fold symmetry and are probably pentamers composed of five molecules of the major capsid protein L1. Reproduced by kind permission from Stannard, 1997.

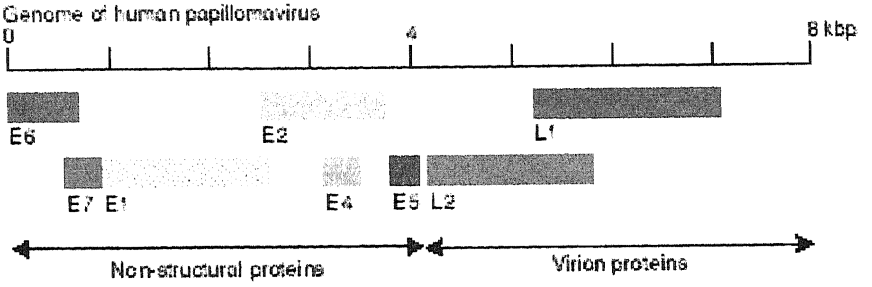


Figure 1.4 HPV genomic structure. Reproduced with permission Cambridge University Press (Man, 1998).
Simplified organisation (linearised) of the human papillomavirus type-16 genome. Scale bar is in kilobase pairs. Rectangles represent the positions of open reading frames. The E genes encode proteins that are produced early in the infectious cycle (non-structural proteins); the L genes encode proteins that are produced late in infection (necessary for virus assembly). The actual protein products are complex owing to the production of multiple messenger RNA transcripts, (see section 1.3.3).

1.2.2 Genome organisation of human papillomavirus type-16

1.2.2.1 Non coding region (NCR)

The NCR (also known as the upstream regulatory region and the long control region) of HPV-16 extends from the end of the late region to the beginning of the early region (Fig 1.4). The NCR spans approximately 1,000 bp and is a poorly conserved region present in the integrated regions of HPV in host DNA in cervical cancers. The NCR

is divided into three segments by four E2 binding sites, from the 5' end, the first and second E2 binding sites divide the NCR into three functionally distinct segments. The 5' segment is approximately 300 bp long and lies between the L1 termination codon and the first E2 binding site. The E2 binding site influences transcription from the E6/E7 promoter (Romanczuk *et al.*, 1990). This segment contains transcription termination and polyadenylation sites for late transcripts as well as negative regulatory elements (Kennedy *et al.*, 1991).

The central segment of the NCR is approximately 400 bp long and is flanked by two E2 binding sites (Gloss *et al.*, 1987). This segment functions as an epithelial-specific transcription enhancer which may control the epithelial tropism of HPVs (Gloss *et al.*, 1987; Cripe *et al.*, 1987). This enhancer is modulated by physiological factors including steroid hormones and intracellular signalling pathways downstream of membrane bound receptors. At least nine different cellular transcription factors have been reported to bind about twenty different sites on this segment. These factors include, activator protein (AP1; Chan *et al.*, 1990), papillomavirus enhancer binding factor (PEF1; Cuthill *et al.*, 1993), glucocorticoid and progesterone receptors; (Chan *et al.*, 1989), nuclear factor (NF1), octamer binding factor 1 (Oct-1; Sibbet *et al.*, 1995), transcriptional enhancer factors (TEF-1 & TEF-2; Chong *et al.*, 1991; Ishiji *et al.*, 1992) and the transcription repressor (YY-1) yin yang-1 (Gloss *et al.*, 1987; 1989; Chong *et al.*, 1990; Sibbet & Campo, 1990; Bauknecht *et al.*, 1992).

The 3' segment is approximately 140 bp long and contains the E1 binding site, which identifies the origin of replication. This segment also contains a Sp1 transcription factor binding site, two E2 binding sites and a TATA box.

1.2.2.2 The L1 protein

The amino acid sequences of the L1 proteins are highly conserved amongst all papillomaviruses (PVs). They have an average molecular mass of 55,000 Daltons (Da) and are partially glycosylated which is unusual for proteins that are localised to the nucleus (Larsen *et al.*, 1987; Browne *et al.*, 1988; Zhou *et al.*, 1990; Iftner *et al.*, 1990). By using vaccinia virus or baculovirus expression systems, L1 alone, forms virus like particles in mammalian cells (Hagensee *et al.*, 1993; Kirnbauer *et al.*, 1992; Rose *et al.*, 1993; Zhou *et al.*, 1991; see 1.4.2).

1.2.2.3 The L2 protein

The L2 proteins have a molecular mass of 76,000 Da and are not as conserved as the L1 proteins (Doorbar & Gallimore, 1987; Komly *et al.*, 1986). Recombinant L1 capsids expressed in mammalian cells *via* vaccinia virus appear to be identical to capsids of recombinant L1/L2 expressed the same way although capsid formation with L2 is more efficient (Hagensee *et al.*, 1993). This suggests that L2 may play a role in virus assembly. As the amino terminus is able to bind DNA, it is thought that L2 has a role in attracting viral DNA during the assembly of viral particles or in the positioning of the minichromosome within the capsid (Zhou *et al.*, 1994).

1.2.2.4 The E1 protein

Depending on HPV type, E1 proteins have a molecular weight of 68,000-85,000 Da and are between 600 and 650 amino acids long (Zhou *et al.*, 1994). The E1 protein accounts for the only enzymatic activities associated with PVs, namely the coupled NTPase and helicase activities. E1 may be described as a DNA-dependent ATPase or

an ATP-dependent helicase and is essential for episomal replication of the viral genome, interacting with DNA in a sequence independent manner, binding to the origin of replication and unwinding DNA at the replication fork (Seo *et al.*, 1993; Yang *et al.*, 1993). It also engages in a variety of important interactions with E2, host replicative mechanisms and with itself undergoing multimerisation.

1.2.2.5 The E2 protein

As a result of mRNA splicing HPV-16, like other HPVs, encodes two E2 proteins. The full length E2 protein is approximately 43,000-48,000 Da and consists of three functional domains. These are a trans-acting domain in the amino terminus approximately 200 amino acids long, a flexible hinge which varies in size and sequence among PVs which overlaps the E4 gene in a different reading frame; and a DNA binding and protein dimerisation domain in the 90 carboxyl terminal residues (Giri & Yaniv, 1988; McBride *et al.*, 1988). The E2 protein is involved in transcriptional regulation and viral replication by binding as a dimer to a palindromic consensus sequence $ACCN_6GGT$ known as the E2 binding site or the E2 responsive sequence (Androphy *et al.*, 1987; McBride *et al.*, 1988; Hirochika *et al.*, 1988; Chiang *et al.*, 1991; Ustav & Stenlund, 1991; Winokur & McBride, 1992). There are multiple copies of E2 binding sites in all PVs and some of these are critical for the activity and regulation of the viral origin of replication.

1.2.2.6 The E4 protein

HPV-16 E4 is a 10,000 Da protein and is detectable in the upper spinous and granular layers of epithelium only (Seedorf *et al.*, 1987; Stoler *et al.*, 1989; Crum *et al.*, 1990).

E4 is generally expressed at much lower levels in mucosal infections than in cutaneous infections and is usually cytoplasmic but can be found associated with cellular membranes or localised to the cell nucleus (Crum *et al.,* 1990; Tomita *et al.*, 1991; Palefsky *et al.*, 1991; Doorbar *et al.*, 1992).

HPV-16 E4 is involved in disruption of keratin filaments in keratinised epithelium and expression of E4 in some cell lines results in disruption of the cytokeratin intermediate filament (Sterling *et al.*, 1993; Doorbar *et al.*, 1996; Roberts *et al.*, 1997). It is thought that one of its functions may be to prevent the cornification of squamous epithelia facilitating the release of viruses once the cells are shed (Doorbar *et al.*, 1996).

1.2.2.7 The E5 protein

Almost all HPV types, exceptions being the epidermotropic HPV types -5 and -8, encode the E5 protein. HPV E5 proteins are poorly conserved but all share an extremely hydrophobic structure with three transmembrane regions. HPV-16 E5 interacts with the 16,000 Da proton pump protein and has been shown to interfere with the degradation of epidermal growth factor receptors (EGF-R; Straight *et al.*, 1993). It has also been shown to exhibit weak transforming activity in immortalised fibroblasts, inducing anchorage independence (Leechanachai *et al.*, 1992; Pim *et al.*, 1992).

1.2.2.8 The E6 protein

HPV E6 is encoded by a major oncogene of approximately 150 amino acids. E6 contains several zinc finger motifs Cys-X-X-Cys and appear to represent duplication

of the carboxyl-terminal region of E7 (Grossman & Laimins, 1989; Barbosa *et al.*, 1990). The E6 product of HPV-16 and -18 has been localised to the nucleus and the cysteine motifs appear to be important for the nuclear translocation since mutants lacking this motif localise predominantly to the cytoplasm (Grossman & Laimins, 1989; Kanda *et al.*, 1991).

Similar to proteins encoded by other cancer causing viruses, for example, the E1B protein of adenovirus and the large T antigen of SV40, E6 is associated with the tumour suppresser gene p53 (Sarnow *et al.*, 1982; Werness *et al.*, 1990; Scheffner *et al.*, 1990). This association is mediated by certain risk HPV types only and results in the degradation of the p53 protein mediated through an interaction with the 100,000 Da E6-AP cellular protein (Werness *et al.*, 1990; Huibregtse *et al.*, 1993). HPV-16 E6 is able to transactivate a series of promoters that activate telomerase in a p53 independent pathway to overcome p53 mediated G1 arrest and apoptosis (Sedman *et al.*, 1991; Desaintes *et al.*, 1992; Klingelhutz *et al.*, 1996).

1.2.2.9 The E7 protein

The E7 ORF is a second major oncogene that encodes a protein of 100 amino acids. E7 has transcriptional activity and can be found localised within nuclear compartments and in the cytoplasm (Smotkin & Wettstein, 1987; Sato *et al.*, 1989). Like E6, E7 contains zinc binding domains Cys-X-X-Cys which are thought to facilitate the dimerisation of E7 proteins and play a role in protein stability (Watanabe *et al.*, 1990; McIntyre *et al.*, 1993).

E7 has distinct sequence homology with two other proteins from DNA virus proteins, namely, adenovirus E1A and SV40 large T-antigen. Like these proteins, E7 is able to

bind the tumour suppressor retinoblastoma gene product (pRB) and thus interferes with cellular growth and cycling (Phelps *et al.*, 1988; Whyte *et al.*, 1988; DeCaprio *et al.*, 1988; Dyson *et al.*, 1989; Imai *et al.*, 1991). The E7 proteins of high-risk HPV types have greater affinity for pRB than the low risk types (Dyson *et al.*, 1989; Münger *et al.*, 1991; Heck *et al.*, 1992; Sang & Barbosa, 1992). The E7 protein also binds some pRB related proteins p107 and p130 and may modulate the cell cycle in other ways for example by binding to cyclin A and cyclin dependent kinase 2 or histone H1 kinase controlling transition from the G1 phase to the S phase of the cell cycle (Dyson *et al.*, 1992; Davies & Vousden, 1992).

1.2.3 Classification of papillomaviruses

PVs are classified according to their host range and the degree of DNA homology (Coggin & zur Hausen, 1979). HPV-1 and bovine papillomavirus type-1 (BPV-1) were the first to be sequenced allowing the genetic organisation of PVs to be determined (Danos *et al.*, 1982; Chen *et al.*, 1982).

The episomal and linear forms of HPV DNA were first characterised in 1963, when it was thought that all 'wart like' lesions in humans were caused by a single type of HPV and that lesions were determined by the anatomical site (Crawford & Crawford, 1963). The concept of more than one HPV was first proposed when five different types were described (Law *et al.*, 1979). Subsequently, a uniform nomenclature was proposed under which a new virus isolate was defined as having less than 50% homology with known PV types using DNA:DNA liquid hybridisation techniques (Coggins & zur Hausen, 1979).

Later, after advances in DNA sequencing, the nomenclature was changed. Under this system, for a new type of PV to be classified its entire genome had to be cloned and the nucleotide sequences of its E6, E7 and L1 open reading frames (ORFs) had to share less than 90% identity to the homologous sequences of other PVs (9^{th} International Conference on Papillomavirus, Seattle, 1991). This classification was changed again at the 13^{th} International Papillomavirus meeting in Quebec, 1995, where a new type of PV can be classified as long as its complete L1 ORF displays less than 90% identity to other L1 sequences. To date over 95 different HPVs have been cloned (van Ranst *et al.*, 1996).

DNA sequencing has also permitted the classification of HPVs into different phylogenetic groups in which viruses with similar tropism or malignant potential are placed together (Chan *et al.*, 1992; 1995: van Ranst *et al.*, 1992; 1996; Figure 1.5). DNA sequence analysis has also allowed the designing of specific or consensus oligonucleotide primers for the PCR mediated detection of HPV DNA in clinical samples and has allowed epidemiological and natural history studies of a range of HPV infections (van den Brule *et al.*, 1990; Manos *et al.*, 1992).

1.2.4 Subtypes, variants and the evolution of human papillomaviruses

Although genetically stable, some intratypic variation does occur leading to subtypes and variants of HPV types. A subtype displays between 90 and 98% L1 sequence similarity and not many of these have been found. A variant is defined as having more than 98% nucleotide similarity to the reference.

As the sequence variance of individual HPV types appears clustered in different geographical regions of the world it has been possible to study the spread and

evolution of these variants in several populations. For example, it is now believed that HPVs originated in Africa, where the largest number of different variants and the deepest branches are found as well as the closest relatives between HPV-18 and -45 (Chan *et al.*, 1992; Eschle *et al.*, 1992; Ong *et al.*, 1993; Tornesello *et al.*, 1997). Further indication that these viruses probably co-evolved with their hosts are the presence of European, Asian and Native American phylogenetic trees (Ho *et al.*, 1993; Ong *et al.*, 1993).

Studies now indicate that HPV types -16 and -18 have evolved in a slow accumulation of mutations and have co-migrated as stable variant lineages with their human hosts over several hundred thousand years (Icenogle *et al.*, 1991; Ho *et al.*, 1991; 1993; Chan *et al.*, 1992; Schwartzman *et al.*, 1993; Ong *et al.*, 1993).

Three non-human primate PVs closely related to HPVs have also been identified. These are the Rhesus monkey papillomavirus (RhPV-1), related to HPV-11 and –16: the Colobus Guereza monkey papillomavirus-1 (CgPV-1), related to HPV-3 and -10 and the Pygmy Chimpanzee papillomavirus (PcPV), related to HPV-13 (Ostrow *et al.*, 1991; Chan *et al.* 1992; van Ranst *et al.*, 1992), suggesting a common ancestor.

Of the 95 types of HPV more than 30 infect the genital tract, HPV-6 and-11 are found in virtually all benign genital warts (CA) and occasionally in low grade SIL (van Ranst *et al.*, 1996). In contrast, HPV-16 (and also, but less frequently, HPV types-18, -31, -33, -35, -51 & -54) is found in up to 90% of cervical cancers and the majority of SIL (Resnick *et al.*, 1990; van den Brule *et al.*, 1991; zur Hausen, 1991).

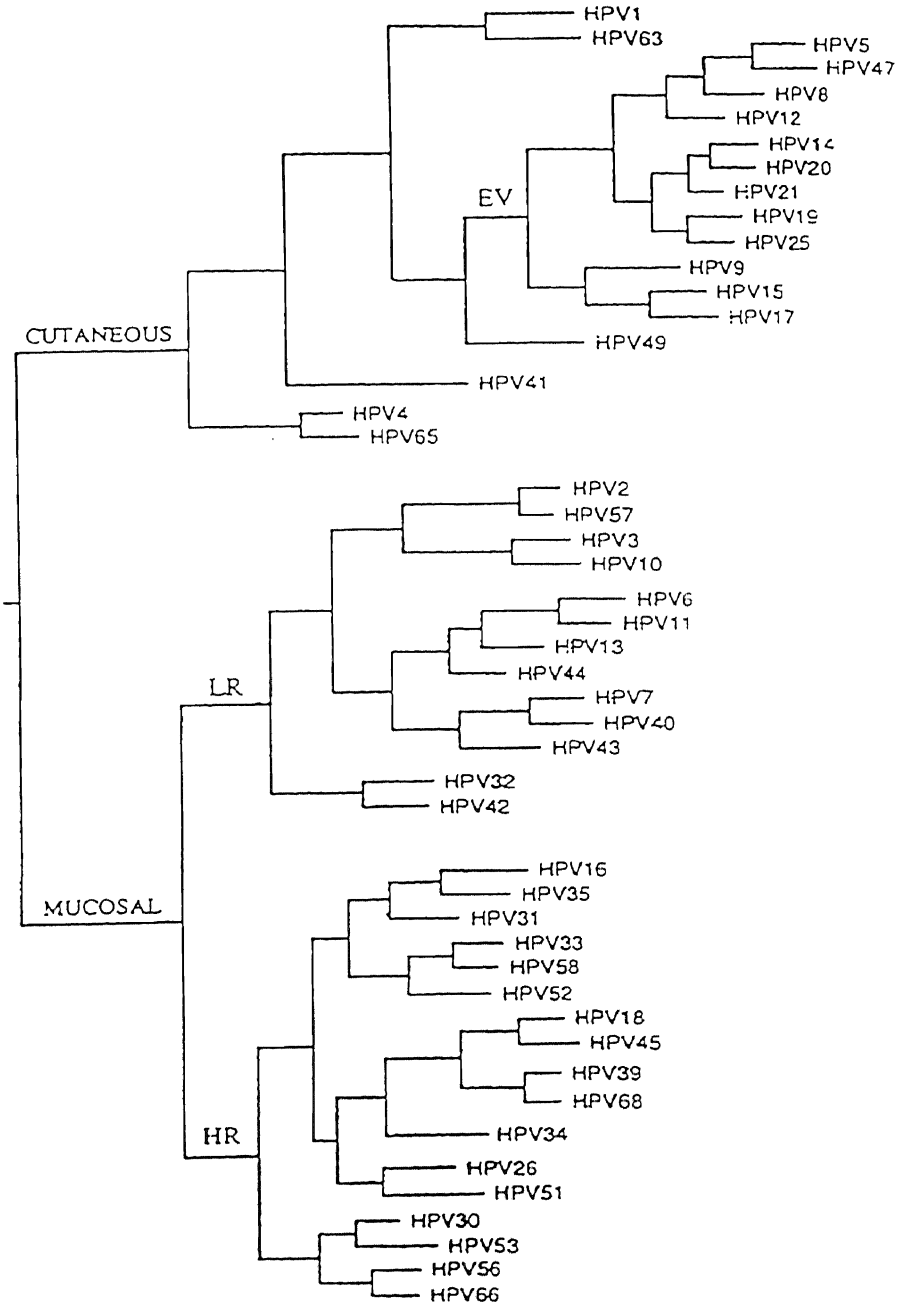


Figure 1.5 Phylogenetic tree; constructed from the alignment of 384 nucleotides in the E6 gene of 48 HPV types. The phylogenetic tree divides HPVs into groups that are primarily associated with cutaneous and mucosal lesions but also into high and low risk mucosal types. The high risk and low risk classification of HPVs correlates well with the *in vitro* biological activity of the E6 and E7 genes Reproduced with kind permission from Professor Marc van Ranst (1996). **LR**=low risk, **HR**=high risk.

1.3 HUMAN PAPILLOMAVIRUS INFECTIONS

HPV gains entry through micro-abrasions and enters basal cells by direct binding to cell surface ligands which belong to the α_6-intergrin family (Evander *et al.*, 1997). The HPV life cycle is tied to the differentiation of the keratinocyte and can be split into two stages, non-productive and productive. In the non-productive stage the viral genome is established as a low copy number nuclear plasmid. This occurs in the proliferating basal layer of the epithelium where HPV replicates its DNA to keep up with the division of basal and parabasal cells and establishes a steady state level of viral genomes (Chow & Broker, 1994). As infected cells undergo their normal lifecycle a subset of daughter cells become detached from the basement membrane to stratify and differentiate. This is where the productive stage of the viral life cycle begins in the terminally differentiated layers of epithelium. During the productive stage, HPV amplifies its genome to a higher copy number, expresses late genes encoding the capsid proteins, and produces viral progeny.

1.3.1 Transmission of human papillomaviruses

The sexual habits of each individual are major risk factors for infection as the same HPV types are not always found in regular male and female partners (Rotola *et al.*, 1995). The prevalence of HPV infection is higher amongst sexually active women and very low in virgins (Karlsson *et al.*, 1995). Infection is believed to be transient in some women and may be associated with a new sexual partner (Evander *et al.*, 1995). Sexual acquisition of HPVs is thought to be dependent on a large reservoir of infected males; men suffer from transient infections which are not usually apparent and have a

low propensity to progress to cancer (Campion *et al.*, 1988; Obalek *et al.*, 1986; Wickenden *et al.*, 1988).

The epidemiology of HPV infection in men has been hampered by the lack of a satisfactory counterpart to the cervical scrape, although, it is possible by applying urethral swabs to the distal 1 centimetre (cm) of the urethra to obtain mucosal epithelial cells (Wikstrom *et al.*, 1991). CA usually occurs in this region, which is thought to be analogous to the transformation zone acting as a reservoir for HPV infection (Oriel, 1990; Omar *et al.*, 1991). The male role in transmission of HPV was investigated after it was determined that monogamous women had higher incidences of cervical cancer, if their male partner had either penile cancer, a number of sexual partners or had previously been married to a woman who had died of cervical cancer (Rotkin, 1967). It is thought that, the male distal urethra may be the major reservoir of genital HPV infection (Siegel & Mellinger, 1992; Higgins *et al.*, 1992).

Studies of HPV infection in children have reported the presence of HPV DNA and HPV specific antibodies in children from 0-13 years old (Sedlacek *et al.*, 1989; Smith *et al.*, 1991; Koch *et al.*, 1997; af Geijersstam *et al.*, 1999). Genital HPVs are not only acquired by sexual contact, but may also be passed from mother to infant or between individuals without physical contact *via* infected clothing, fomites and urine (Ferenczy *et al.*, 1989; Hillman *et al.*, 1993; Fredericks *et al.*, 1993; Armbruster-Moraes *et al.*, 1994; Kaye *et al.*, 1994).

HPV DNA has also been detected in the amniotic fluid, placenta and cervical scrapes of pregnant women with EV, rendering vertical transmission of EV associated HPVs likely (Favre *et al.*, 1998). Indeed, pregnant women with latent HPV infections have been shown to have a 30% transmission rate of transmitting HPV to the

oropharyngeal mucosae of their infants (Tanti *et al.*, 1999). Similarly, another member of the papovaviridae family, JC polyomavirus has been shown to be transmitted from mother to child, while both vertical and horizontal transmission of BPV-2 has been described under experimental conditions in cattle (Kunitake *et al.*, 1995). PVs are stable to heat and desiccation. HPV-16 DNA has even been isolated from surgical instruments and in smoke generated by the laser ablation of lesions (Smotkin, 1989; Kashima, 1991).

1.3.2 HPV infection and the development of malignant lesions

The biological behaviour of HPV is complex but the process involves regression, persistence and progression of the disease (Syrjanen, 1994). HPV detection techniques indicate that transient genital infection is common among asymptomatic women but is insufficient for cervical cancer development. Factors additional to HPV infection are more than likely to be involved in the development of cervical cancer and this fits in with the theory of multi-step progression of carcinogenesis (Farber, 1984). Such additional factors would include: viral integration into the host DNA; activation of cellular oncogenes; inactivation of tumour suppresser genes and failure of the immune system. It is unlikely that HPV infection alone is sufficient for malignant transformation and reasons supporting the multi-step progression of cancer include a long latency period between initial HPV infection and the eventual development of cervical cancer (zur Hausen, 1986). Further, keratinocytes immortalised *in vitro* with high risk HPV types are initially non-tumourigenic when injected into nude mice (Hawley-Nelson *et al.*, 1989; Munger *et al.*, 1989) and

malignant tumours are only evident after prolonged culturing (62 passages or more) of the transformed cells prior to injection (Hurlin *et al.*, 1991).

1.3.3 Human papillomavirus integration into genomic DNA

As previously discussed, it is presently postulated that following viral integration into the host genome a series of molecular events occur (see section 1.3.2). Integration into the host genome occurs when the E1 and E2 genes are inactivated and the production of the E6 and E7 proteins are increased, these events are necessary for viral replication but when over-produced result in the development of cancer, after the functional inactivation of p53 and/or pRB tumour suppressor genes (Schneider-Manoury *et al.*, 1987). These tumour suppressor genes may play a critical role in programmed cell death, which is crucial to the elimination of cells with oncogenic potential (Levine & Momand, 1990).

The site of HPV-16 integration may be random although specific integration into chromosome 8q21-q22, adjacent to c-*myc*, has been described (Durst *et al.*, 1985; 1987; Smotkin & Wettstein, 1986). Once integrated HPV 16 is non-replicating and non-infectious and results in the linearisation of viral DNA with the L1 and L2 ORFs often lying upstream from the viral promoter and unlikely to be transcribed (Gissman & Schwartz, 1986). This permits only the E6 and E7 open reading frames to be transcribed and in carcinomas the mRNA of these ORFs is abundant (Schwartz *et al.*, 1985; Durst *et al.*, 1985; zur Hausen, 1991).

The complex regulation of gene expression in HPVs is controlled by cellular and viral transcription factors, different promoter usage, differential splicing differential transcription termination and mRNA stability. Various splicing patterns and the

activity of different promoters results in a wide range of HPV-16 mRNAs (Smotkin *et al.*, 1989; Rohlfs *et al.*, 1991; Sherman *et al.*, 1992). These mRNAs contain ORFs with translation initiation codons and it is thought that translation of these polycistronic messages is influenced by alternative splicing.

The viral transcription patterns vary considerably in different layers of epithelium; there is weak expression of early genes in the basal layers of low grade cervical dysplasias induced by HPV-16 or -33 and in some induced by HPV-6 or -11 CA (Beyer-Finkler *et al.*, 1990; Durst *et al.*, 1992; Stoler *et al.*, 1992). The majority of HPV-6 containing CA display clear E6 signals in the basal layer and E7 signals in the lowest layers of the epithelium. It is thought that this may be due to evolution of the high risk types in order to prevent neoplastic conversion of the host cells which would be counterproductive for the replication of the virus (Fuchs & Pfister, 1994).

Genes with rare triplet codons are not efficiently translated in undifferentiated mammalian cells and this is manipulated by PVs. In terminally differentiated epithelial cells, the use of rare triplet codons by PVs can result in the efficient expression of late genes as cellular mRNAs are not present in large enough numbers in terminally differentiated cells to compete with PV mRNAs.

HPV integration could also result in the activation of cellular proto-oncogenes which may be a major step towards the malignant transformation of a HPV infected cell. There is an over-expression of the nuclear proto-oncogene *ets*-2 in cells derived from cervical cancer (Dipaolo *et al.*, 1993). The *ras* and *myc* oncogene families have also been shown to be altered in some cervical lesions and this could be a requirement in the progression of cervical cancer (Ocadiz *et al.*, 1987), for example, the amplification of *c-myc* which occurs in HPV transformed cell lines affecting the

morphological and growth characteristics of such cells (Crook *et al.*, 1990). However, the amplification of *c-myc* appears to be a late rather than an initiating event in anogenital malignancy (Crook *et al.*, 1991). Mutations of Ha-*ras* genes have been found in HPV infected cultured keratinocytes and these have been shown to be sufficient to induce complete cellular transformation (Balmain, 1985). However, injection of transformed cells into mice results in the formation of malignant tumours after prolonged culturing indicating that additional factors are required in the pathogenesis of cervical cancer (zur Hausen, 1991; Hurlin *et al.*, 1991).

1.3.4 Other human papillomavirus associated lesions

Genital warts (CA) consist of localised epithelial hyperplasia and unlike cervical lesions they do not progress to malignancy but have a defined boundary and an intact basement membrane. They vary widely in appearance and location and can be divided into genital (mucosal) and non-genital (cutaneous) types, all of which usually regress after a period of time.

Cutaneous EV carcinomas developing in adult life are usually confined to sun exposed sites suggesting a co-carcinogenic role for ultra violet radiation. EV is characterised by impaired cell mediated immunity (CMI) and the appearance in childhood of extensive refractory plane warts mainly over the face and limbs that progress to squamous cell carcinoma in 30-59% of patients. At least 20 HPV types have been found in EV lesions although only a few types (HPV-5 and HPV-8) have consistently been detected in EV malignant tumours (Orth, 1987). It serves as a model not only for cutaneous viral oncogenesis but also of local defence mechanisms in the progression of HPV associated cancer (Lutzner, 1978). Patients with EV are

usually infected with multiple HPVs, which can be classified into two groups based on DNA homology. EV specific transforming HPVs have also been found in warts, pre-malignant and malignant skin lesions of renal transplant recipients (de Jong-Tieben, 1995). The mechanism of EV HPV induced transformation of keratinocytes is unknown but the process seems to differ from that involved in HPV associated cervical cancer in that no integration of viral sequences into the host genome occurs and the effect of EV oncoproteins on tumour suppresser genes is unknown (Androphy, 1994).

HPVs are also known to infect oesophagus, upper and lower respiratory organs, vulva, vagina, bladder and skin. Respiratory papillomas are more common in children than in adults and are associated with HPV types-6 and -11, these can develop into malignant lesions after X-ray irradiation. HPVs may even be involved in a minority of prostate cancers (Dillner *et al.*, 1997). The high risk HPV types -16 and -33 can also be found as rare periungual infections (Moy *et al.*, 1994; Ruedlinger *et al.*, 1989). Cancer of the vagina and vulva is mostly observed in women in their 70s and 80s and has been found to be linked with pre-existing or co-existing HPV-associated genital warts and to seropositivity to HSV-2 (Sherman *et al.*, 1991).

For HPVs, mucosal types affect the anogenital and aerodigestive tract while cutaneous types predominantly infect cutaneous epithelium and are rarely found at anogenital sites or within the oral mucosa. However, there is at least one exception, namely HPV-2, which can cause warts on both the hands and anus (de Villiers *et al.*, 1986; 1991; Fleming *et al.*, 1987; Snijders *et al.*, 1992).

Most animal PVs are associated with the development of squamous epithelial warts, which are either cutaneous or mucosal in origin. A subgroup of PVs are also known

to cause benign fibropapillomas, these proliferative lesions differ from the more usual papillomas in that there is a dermal fibroblastic component associated with squamous epithelial proliferation. This subgroup comprises BPV-1, -2 and -5, European elk papillomavirus, deer papillomavirus and reindeer papillomavirus (Lancaster & Olson, 1982).

Generally PVs are unable to cross species barriers. There are however, two exceptions to this, namely BPV-1 and -2 which can also infect horses, donkeys and sheep although infection in horses is non productive (Segre *et al.*, 1955; Sundberg, 1987; Lancaster, 1981; Angelos *et al.*, 1991; Reid *et al.*, 1994; Trenfield *et al.*, 1990). To date PVs or their antigens have been detected in humans, primates, cattle, rabbits, goats, sheep, horses, dogs, elephants, multimammate rats, wild European harvest mice, molluscs and racoons (Davies & Kemper, 1936; Fulton *et al.*, 1970; Gibbs *et al.*, 1975; Sundberg *et al.*, 1986; 1987a; 1987b; 1988; Amtmann & Wayss, 1987; Kloster *et al.*, 1988; Hamir *et al.*, 1995).

1.3.5 Co-factors

HPVs are not unique amongst PVs with respect to their association with cancers, a number of PVs are strongly associated with the development of malignant lesions in cattle and cottontail rabbits (Shope & Hurst, 1933; Jarrett *et al.*, 1978). In such cases it seems that co-factors are required for malignant transformation, for example, exposure to sunlight acts as a co-carcinogen in the case of patients with EV infected with HPV-8, bracken ingestion in cattle infected with BPV-4 and the application of coal tar in rabbits infected with CRPV (Orth *et al.*, 1978; Jarrett *et al.*, 1978). Indeed,

as discussed below, many endogenous and exogenous factors are strongly associated with the progression of SIL to cancer in humans.

1.3.5.1 Sexual behaviour

The risk of acquiring HPV in the lower genital tract and developing cervical cancer increases with the number of sexual partners independently of other risk factors (Rosenfeld & Wentz, 1989; Moscicki *et al.*, 1990; Fisher *et al.*, 1991; Ley *et al.*, 1991). Indeed, the risk associated with having had sexual intercourse with 10 or more partners is nearly 3 times higher than that associated with one or no partners (Peters *et al.*, 1986; Brinton *et al.*, 1987).

Coitus before the age of 16 years is also an independent risk factor. The odds ratio for developing cervical cancer in subjects who had their first coitus before 16 years of age is 4.3 when compared to those who had remained virgins until 24 years of age (Brinton *et al.*, 1987; Cuzick *et al.*, 1990; Herrero *et al.*, 1990; Bosch *et al.*, 1992). This enhanced susceptibility of the cervix to carcinogenesis may arise due to the development of metaplastic epithelium at the transformation zone that occurs in adolescence (Reid & Campion, 1989). It is also possible that the number of sexual partners and early coitus may only increase the risk of HPV infection (Schiffman *et al* 1993) if barrier contraceptives are not used (Kjaer *et al.*, 1996).

1.3.5.2 Age

An age less than or equal to 30 years has also been shown to be a significant risk factor for HPV infection (van den Velde & van Beers, 1992; Nindl *et al.*, 1997). The group with the highest incidence of HPV infection (13%) is 20-24 years of age and

this is reduced to 5% in those of 55 years of age (de Villiers *et al.*, 1987). Similarly, the falling incidence of cervical cancer in Bombay over the last two decades has been attributed to older age at marriage and a decline in the number of sexual partners (Yede *et al.*, 1989).

1.3.5.3 Diet

Evidence for deficiencies of vitamins A, C, β-carotene and folate have been found for women with invasive cancer and SIL (Harris *et al.*, 1986; Palan *et al.*, 1988). Retinoic acid treatment of HPV-18 containing HeLa cells (derived from cervical cancer) has been found to inhibit E6 and E7 expression suggesting that insufficient retinoic acid receptor gene expression could contribute to cervical cancer (Bartsch *et al.*, 1992). Similarly, animal and *in vitro* studies have shown that retinoic acid can inhibit cell transformation in BPV containing mouse cells and significantly enhances the regression of cottontail rabbit papillomavirus (CRPV)-induced lesions (McMichael, 1965; Tsang *et al.*, 1988).

1.3.5.4 Cigarette smoke

The constituents of tobacco inhalate such as nicotine and cotinine have been found in the cervical mucus of smokers and are thought to have transforming effects on HPV-infected tissue (Hoffmann *et al.*, 1985; Ho *et al.*, 1998). In addition a decreased number of Langherhans' cells is found in the cervical tissues of smokers which may reduce the local cellular immune response. Indeed nicotine derivatives have been isolated from cervical mucus (Schiffman *et al.*, 1987; Barton *et al.*, 1988).

1.3.5.5 Oral contraceptives

Progesterone and glucocorticoid endocrine steroid hormones in the oral contraceptive pill, appear to slightly increase the risk of developing cervical cancer in long term users, and a similar trend has been observed for CA (Francheschi *et al.*, 1983; Daling *et al.*, 1986; Brinton *et al.*, 1986; Peto & zur Hausen, 1986; von Knebel Doeberitz *et al.*, 1990; Pater *et al.*, 1990; Mittal *et al.*, 1993). Treatment with progesterone enhances HPV gene expression which may lead to increased viral production and proliferation of HPV infected cells (Pater *et al.*, 1990). Indeed, infection with HPV has been detected with increasing frequency the longer the oral contraceptives are taken, however, this could equally relate to an increasing number of sexual partners (Ley *et al.*, 1991). The contributions of these steroid hormones are supported by the presence of three hormonal responsive elements in the NCR of genital HPVs (Mittal *et al.*, 1993).

1.3.5.6 Immunosuppression

Evidence for a role of the immune system arises from the increase in HPV associated lesions during immunosuppression. Organ transplant recipients receiving immunosuppressive therapy are also known to have an increased incidence of viral warts, premalignant and cancerous skin lesions and show some similarities to patients with EV. 25-50% of renal transplant recipients develop warts 1 year after transplantation, rising to 77-95% after 5 years (Dyall-Smith *et al.*, 1991; Glover *et al.*, 1994). These tend to be common palmoplantar warts in the early post transplant years, followed by numerous plane warts developing predominantly on sun exposed skin some years later.

Clinical genital HPV infections also occur more frequently during pregnancy, when women are immunocompromised, often with regression of lesions after delivery (Woodruff & Petersen, 1958; Oriel, 1971; Cook *et al.*, 1973; Jablonska *et al.*, 1982; Garry & Jones, 1985). An increase in HPV prevalence may predispose to perinatal transmission of the virus. (Schneider *et al.*, 1987; Czegledy *et al.*, 1989; Villa & Franco, 1989).

1.3.5.7 Other infectious organisms

Other infectious organisms may be involved in the development of cervical cancer in HPV infected tissue. Transient infection with HSV-2 in the presence of ongoing chronic infection with high risk HPVs may contribute to the oncogenic process through a 'hit and run' mechanism (Yamakawa *et al.*, 1994; Galloway & McDougall, 1983). HSV-1 infection may also contribute to an increased risk of cancer in the presence of HPV as ICPO protein is able to activate HPV-18 gene expression in cervical cancer cell lines (Gius & Laimins, 1989). Human herpes virus type 6 has also been associated with some SIL and cervical cancers and there is enhanced expression of HPV early proteins and an increased tumourigenicity in mice (Chen *et al.*, 1994).

Other infectious agents that have been suggested to act as co-factors in the development of SIL and cervical cancer include *Treponema pallidum, Neisseria gonorrhoea*, cytomegalovirus and Epstein Barr virus, although no substantive data have been described (Lacey, 1992). Several studies have shown that 20% to 32% of men attending genitourinary clinics with genital warts have evidence of at least one other sexually transmitted disease. For example, the incidence of *Neisseria*

gonorrhoea in these patients can be as high as 9.9% (Kinghorn, 1978; Carne & Dockerty, 1990; Crawshaw & Haran, 1990).

HPV infection of the anogenital tract is more common in subjects infected with human immunodeficiency virus (HIV) due to the immunosuppression that occurs during HIV infection and also as a result of viral interaction and alteration of viral pathogenesis (Palefsky, 1995).

1.3.5.8 Genetic susceptibility

Many diseases are thought to have a genetic basis. There is a significant link between cervical carcinomas containing HPV types-16 or -18 and presence of the human leukocyte antigen (HLA) DQBI*0602 allele. The HLA-II antigen Dqw3 (an allele of the DQBI) is expressed by 67-88 % of women with cervical cancer while only 50% of a control population express this antigen (Wank & Thomssen, 1991; Wank *et al.*, 1992; Helland *et al.*, 1992). Individuals with certain tissue types *e.g.* HLA-B7 tend to have more aggressive carcinomas with a poorer prognosis (Ellis *et al.*, 1995). Conversely, the absence of HLA-B7/B40 has been associated with metastatic spread of HPV-16 containing cervical carcinomas. However, at present, it is not completely clear whether these genetic associations represent susceptibility to cancer development and/or to infection with cancer associated viruses (Wank *et al.*, 1993; Honma *et al.*, 1994).

1.4 LABORATORY INVESTIGATIONS OF HUMAN PAPILLOMAVIRUSES

Studies of HPVs have been hampered as virions are often only present in low amounts in clinical lesions and are notoriously difficult to propagate either *in vitro* or

in vivo. HPV replication occurs in the supra basal layers of squamous epithelium. The expression of early proteins is largely restricted to these layers, while virion assembly takes place in the upper layers where capsid proteins are expressed. This localisation, together with the non-lytic nature of HPV, may provide the initial barrier against the immune system. This may also explain the delay of serum antibody responses to virus like particles (VLPs) in women after the first exposure to HPV and the late induction of T-lymphocytes to E7 (Carter *et al.*, 1996). It appears that HPV infections are ignored until antigen is released due to physical damage (biopsy) or by the over expression of E7 after viral integration (Jeon & Lambert, 1995). This 'immunological tolerance' theory is supported by studies in E7 transgenic mice, although keratinocytes expressed and presented a known E7 epitope cytotoxic T-lymphocytes (CTLs) were neither primed nor tolerised *in vivo*. However, CTLs against this epitope could be primed *in vitro* providing that clonal deletion does not occur, and that consequently there is no possibility of a hole in the T-lymphocyte repertoire of these mice (Herd *et al.*, 1997; Melero *et al.*, 1997).

HPV-11 has been replicated *in vivo* using nude mice with infected human xenografts inserted under the renal capsule (Kreider *et al.*, 1987). Subsequently, HPV-16 virions have also been produced in a single nude-mouse transplanted with the SIL-derived cell line W12 but this has not been repeated (Sterling *et al.*, 1990). Infectious HPV-31 virions were produced *via* HPV-31 DNA infected human keratinocytes propagated using raft cell cultures which permit keratinocyte differentiation at an air/liquid interface (Meyers *et al.*, 1992). Unfortunately, the yield of particles in these systems is low, hence the main source of HPV proteins used for research purposes are those produced by the techniques of recombinant DNA or organic chemistry.

The antigens chosen for investigations in this thesis are synthetically constructed peptides, that have been used successfully in previous studies for investigating HPV proteins (Jocmus-Kudielka *et al.*, 1989; Mann *et al.*, 1990; Mandelson *et al.*, 1992; Muller *et al.*, 1992; Hamsikova *et al.*, 1994). Synthetically constructed peptides have also been used in viral diagnosis to detect serum antibody levels and include: hepatitis delta antigen (Poisson *et al.*, 1993), hepatitis E virus (Coursaget *et al.*, 1994), HPV-16 E2 protein (Sharma *et al.*, 1996), respiratory syncytial virus (Langedijk *et al.*, 1996) and the Japanese encephalitis virus (Huang *et al.*, 1996). Indeed, in mice immunised with synthetic peptides to internal proteins of influenza virus, the peptides are often recognised and lysed by CTLs just as well as target cells that had been infected with live influenza (Townsend, 1986).

1.4.1 Animal models

Animal models used for immunological investigations of natural PV infections may provide parallels for HPV infections and are useful in evaluation of potential vaccination strategies. One of the most useful ways of modelling HPV infection is to examine natural animal infections. For example, there are multiple types of BPV which attack the skin in a similar manner to HPV. Indeed, like 'high risk' HPVs, BPV-4 can also progress to cancer, but the genome is lost in the process. Animals vaccinated with BPV-2 L1 are protected from challenge with BPV-1 or -2 virions but are still susceptible to BPV-4 infections indicating that vaccination induces type-specific immunity (Jarrett *et al.*, 1990). Protection against re-infection *via* neutralising antibodies to BPV-1 & -2 was induced by inoculating cattle with bacterial L1 and L2 fusion proteins (Pilacinski *et al.*, 1986; Jarrett *et al.*, 1991). BPV-

4 E7 fusion proteins can also induce regression of papillomas, presumably through the induction of specific CTLs. Similarly, immunisation of cattle with BPV-2 L2 fusion protein induces regression of benign tumours in cattle (Jarrett *et al.*, 1992).

Similarly, CRPV cause skin papillomas that can spontaneously regress or progress to squamous cell carcinoma. Vaccination of cotton tail rabbits parentally (to avoid contact with skin) with virus, protects against subsequent infection but does not induce regression of established lesions (Shope, 1937). Vaccination of cotton tail rabbits using bacterial fusion L1 or L2 constructs, vaccinia virus expressing L1 or, virus-like particles results in the production of neutralising antibodies and protection to subsequent viral challenge. Vaccination with non-structural proteins E1 and E2 can induce regression of established papillomas (Christensen *et al.*, 1991; Breitburd *et al.*, 1995; Selvakumar *et al.*, 1995).

Canine oral papillomaviruses (COPV) also cause lesions on mucosal surfaces but these do not usually progress to malignancy unless injected intramuscularly (Sundberg *et al.*, 1987). A crude formalin inactivated oral papilloma homogenate was used successfully in a large study to prevent oral COPV infections (Bell *et al.*, 1994). This led to further trials of COPV L1 VLPs in beagles, resulting in production of neutralising antibodies and animals that were completely resistant to re-challenge with COPV. This has now progressed to vaccine trials for high risk HPVs developed by MedImmune (Table 1.1).

Table 1.1 A summary of vaccines currently undergoing clinical trials

Company	Components	Type	Development stage	Reference
Apollon	Plasmid DNA types unknown	Unknown	Pre-clinical	National Cancer Institiute, 1999
Cantab	Live recombinant vaccinia virus, HPV-16 & -18 E6/E7 (TA-HPV)	Therapeutic	Phase I/II	Editorial, The Scientist, 1997
Cantab	Recombinant L2 and E7 of HPV-6 (TA-GW)	Therapeutic	Phase IIa	Lacey *et al.*, 1999
Cantab	Unknown (TA-CIN)	Unknown	Pre-clinical	Editorial, The Scientist, 1997
MedImmune	VLP (L1) of HPV-11	Prophylactic	Phase I	National Cancer Institute, 1999
MedImmune	Multivalent VLPs high risk types	Prophylactic	Phase I	National Cancer Institute, 1999
Merck	VLP (L1) of various HPV types	Prophylactic	Pre-clinical	National Cancer Institute, 1999
Merck	Naked plasmid DNA HPV types unknown	Unknown	Pre-clinical	National Cancer Institute, 1999
NCI	HPV-16 E7 + lipid moiety	Therapeutic	Pre-clinical	National Cancer Institute, 1999
Pangaea	Plasmid DNA encoding HPV MHC epitopes	Therapeutic	Phase I/IIa	Editorial, The Scientist, 1997
Pasteur Merieux Connaught	Vaccinia/molecular tag VLP/E7 chimera	Therapeutic	Pre-clinical	Editorial, The Scientist, 1997
Stressgen	Recombinant E7 (HspE7)	Therapeutic	Pre-clinical	Canadian News Wire, 1997

RhPV positive neoplastic genital lesions have been reported in sexually-active monkeys. These may provide a model for HPV associated cervical oncogenesis as RhPV is closely related to HPV-16 and may have similar oncogenic potential (Ostrow *et al.*, 1990). RhPV-1 E5, E6 and E7 DNA can transform murine NIH 3T3 cells *in vitro* (Ostrow *et al.*, 1993; Schneider *et al.*, 1991).

Transgenic mouse models have proved extremely useful in the study of the transforming properties of PVs. The first transgenic model to be described was for BPV-1 where mice developed dermal fibrosarcomas involving at least three stages mimicking the multi-step development of cancer (Lacey *et al.*, 1986). Transgenic models for HPV also exist and HPV-16 transforming genes have been targeted to a variety of tissues including the skin, where squamous epithelial hyperplasia and dysplasia develop and sometimes progress to cancer (Arbeit *et al.*, 1994).

1.4.2 Virus like particles

Using vaccinia virus vectors, HPV-16 L1 and L2 can be assembled into empty virus like particles (VLPs) in the nuclei of infected cells (Zhou *et al.*, 1991). In HPV types -1, -6, -11, -16, BPV-1 and CRPV, the L1 protein is sufficient to form VLPs but the presence of L2 significantly increases the efficiency of the L1 assembly process (Kirnbauer *et al.*, 1993; Hagensee *et al.*, 1993; Rose *et al.*, 1993; Zhou *et al.*, 1993; Sasawaga *et al.*, 1995; Heino *et al.*, 1995). VLPs display authentic particle morphology and are highly immunogenic probably carrying the same conformational epitopes as native particles (Hagensee *et al.*, 1993; Rose *et al.*, 1993; Zhou *et al.*, 1993). Several workers are now investigating the use of VLPs as prophylactic vaccines or chimeric (prophylactic/therapeutic) vaccines, and it may even be possible

to use VLPs as vectors for delivering HPV DNA vaccines into target cells as with polyomavirus (Slilaty *et al.*, 1982; Slilaty & Aposhian, 1983; Table 1.1).

1.5 CONTROL OF HUMAN PAPILLOMAVIRUSES BY THE IMMUNE SYSTEM

The importance of an effective immune response in the control of HPV infection can be demonstrated by the increase of HPV associated lesions in the immunosuppresed (see section 1.3.5.6). This section describes immune responses to HPV infection and the methods used to detect them. Also included are protocols for vaccine development, some of which are still experimental while others are undergoing clinical trials.

1.5.1 An overview of immune responses to viruses

Both antigen specific and innate immunity are important in the control of virus infections. Indeed, natural killer (NK) cell activity is associated with the regression of HPV-induced cervical lesions (Garzetti *et al.*, 1995). However, it is antigen specific immunity that can specifically target responses and immunological memory.

Mechanisms used to eliminate virus infections include viral neutralisation, where antibodies specific for virus surface antigen contain the spread of acute infection and protect against re-infection. In the context of HPV infection, secretory immunoglobulin class A (IgA) in mucous secretions may play an important role in host defence by blocking viral attachment to mucosal epithelial cells. Circulating antibody or complement may also agglutinate and oppsonise virus particles facilitating Fc or C3b receptor mediated phagocytosis. Once an infection has

occurred, antibodies are not usually able to eliminate a virus, particularly if the virus is capable of entering a latent state in which its DNA is integrated into host chromosomal DNA. The main components of a cell mediated immune (CMI) response are clusters of differentiation antigen 8 (CD8+) positive CTLs and CD4+ T helper type 1 cells (Th_1). Activated Th_1 cells produce cytokines including interferon gamma (IFN-γ) and interleukin-2 (IL-2). IFN-γ acts directly to eliminate virus by inducing an anti-viral state in cells while IL-2 acts indirectly by assisting the activation of CTL precursors into an effector population. Both IFN-γ and IL-2 activate natural killer (NK) cells, which are important in the first few days of infection until a specific CTL response develops. In most viral infections a CTL response develops within 3-4 days. As CTLs destroy infected cells, they eliminate potential sources of new infection.

HPV infections resemble those of non-lytic viruses as they do not cause cell death but are released from infected cells by desquamation (Stanley, 1994). Therefore, the ideal defence against these infections would be a combination of neutralising antibodies and CTL mediated cell lysis of infected cells for the prevention of subsequent re-infection by released virions (Zinkernagel, 1996). The target cell for CTL mediated lysis would be the keratinocyte present in the intermediate layers of the squamous epithelium where viral transcription and replication takes place and where the early proteins (E1, E2, E4, E5, E6 & E7) are abundantly expressed. The late proteins L1 and L2 would be unsuitable CTL targets as they are expressed in the superficial layers where cells are already dying. Neutralising antibodies would have to be directed at these proteins (Schiller & Roden, 1995).

1.5.2 Antibody responses in human papillomavirus associated lesions

Seropositivity to HPV-16 may be associated with persistent infection, as 83% of women found to be HPV-16 DNA positive on more than one occasion had detectable HPV antibodies compared to 22% for those found to be HPV DNA positive only once (Wideroff *et al.*, 1995). Generally, 50-60% of women who are positive for HPV-16 DNA have circulating antibodies to HPV-16 VLPs. When lesions clear, however, antibody responses decline rapidly (Heim *et al.*, 1995; Wikstrom *et al.*, 1995; Andersson-Ellstrom *et al.*, 1996). Patients infected with genital HPVs often exhibit immune responses as antibodies, for example, HPV-16 E4 antibodies occur in sera from immunocompromised patients and from patients with H-SIL and these may even be predictive of HPV infection (Jochmas-Kudielka *et al.*, 1992; Muller *et al.*, 1990). Moderate reactivity has been detected in the sera of HPV-16 DNA positive SIL patients to HPV-16 L1 synthetic peptides and VLPs (Cason *et al.*, 1992). Naturally occurring neutralising antibodies to HPVs are type-specific and recognise conformational epitopes (Steele & Gallimore, 1990; Jarrett *et al.*, 1984; Christensen *et al.*, 1990; Jensen *et al.*, 1980; Pfister *et al.*, 1981; Favre *et al.*, 1975). Responses to VLPs are more often detected in subjects with genital warts than those without (Heim *et al.*, 1995). Viral persistence is thought to be a key factor in disease progression as increased HPV-16 seropositivity is associated with an increased risk of developing cervical cancer (Nonnenmacher *et al.*, 1995; Lehtinen *et al.*, 1996).

1.5.3 Cell mediated immunity in human papillomavirus-associated lesions

It is CMI which is thought to play a major role in containing HPV infections, and reasons for this include:

(i) Human warts are more prevalent and increase in size during conditions that suppress T-lymphocyte functions, these include pregnancy, immunosuppressive chemotherapy, organ transplantation and HIV infection (see section1.3.5.6). These warts tend to disappear when the immunosuppression is reduced or eliminated. The regression of papillomas in rabbits and cattle is also thought to occur by CMI (Spradbrow, 1987).

(ii) The histological picture of regression flat skin warts shows infiltration by mononuclear cells suggestive of a CMI response (Tagami *et al.*, 1983). Trauma to one wart often results in the regression of warts at other sites, suggesting an immunological response triggered by release of antigen from the wart.

(iii) An altered CD4+/CD8+ ratio has been reported in patients with extensive genital HPV infections (Carson *et al.*, 1986).

(iv) Genital cancers associated with HPVs occur at increased frequency in organ transplant recipients (Penn, 1986).

CMI, rather than humoral immunity, appears to be of importance against both *de novo* and re-infection with HPVs. Patients with common variable immunodeficiency (characterised by the inability to produce specific-antibodies, but intact T-lymphocyte responses) are not unduly susceptible to the development of HPV lesions (Benton *et al.*, 1992). Conversely, CMI responses probably explain why most SIL do not progress to malignancy since inherited, iatrogenic or acquired immunosuppression encourages development of lesions and progression to cancer (Nasiell *et al.*, 1976). In patients with severe H-SIL and cervical cancer HPV-16 and -18 E6/E7 specific CTLs are present in the peripheral blood and at sites of disease implying that CMI is triggered eventually (Borysiewicz *et al.*, 1997). These findings have led to phase II

clinical trials of a live recombinant vaccinia virus expressing the E6 and E7 proteins of HPV-16 and -18 (TA-HPV; [see section 1.6, Table 1.1]).

Th cells can be divided into two subsets according to cytokine expression and immunoregulatory function: Th_1 cells promote predominantly CMI while Th_2 cells stimulate humoral immunity (Mosmann & Sad, 1997). The current prevailing dogma in viral immunology is that Th_1 type CMI is vital to the clearance of infection while Th_2 activity is detrimental and leads to viral persistence. However, there is an increasing awareness that successful immunity against pathogens most often does not entail polarised Th_1 or Th_2 responses but usually more subtle combinations of the two (Allen & Maizels, 1997).

CD4+ Th cells are also important, as they are required for the potent anti-viral and anti-tumour immune responses and provide long term immunological memory (Chen & Ananthaswamy, 1993; Sprent, 1994; Lin *et al.*, 1996). CD4+ Th cells may also play an important role in the elimination of HPV infection. Evidence for this includes (i) regressing warts are mainly infiltrated by CD4+ Th cells, reminiscent of a delayed type hypersensitivity (DTH) reaction (Rogozinski *et al.*, 1988; Vardy *et al.*, 1990; Coleman *et al.*, 1994); (ii) in mice grafts of E7 transfected keratinocytes on skin led to priming of specific CD4+ T-lymphocyte which were detectable after challenge in a DTH reaction (McLean *et al.*, 1993); (iii) vaccination of mice with HPV-16 E7-LAMP-1 vaccinia virus construct targeted E7 to lysosomal and endosomal compartments in the major histocompatiblity complex class II (MHC-II) pathway led to enhanced protection against subsequent challenge (Lin *et al.*, 1996; Wu *et al.*, 1995); (iv) HLA associations with susceptibility to or protection from HPV infection and cervical disease have been found for the MHC-II HLA-DR and HLA-DQ alleles

(Wank *et al.*, 1991; 1993; Apple *et al.*, 1994; 1995; Odunsi *et al.*, 1996; Sastre-Garau *et al.*, 1996).

1.5.4 Immunological cells in human papillomavirus infected lesions

Both cervical cancer and SIL are infiltrated with poorly activated CD8+ T-lymphocytes that show very little *in vitro* tumour specific activation of CTLs and only a few express IL-2 receptors (Ferguson *et al.*, 1985; Viac *et al.*, 1990; Glew *et al.*, 1992; Ghosh & Moore, 1992). A reduction in the number of the professional antigen presenting Langherhans' cells is also seen in genital warts and L-SIL (Viac *et al.*, 1990). This is more pronounced in H-SIL and the histology of Langherhans' cells in these lesions is often disturbed showing abnormal morphology (Hughes *et al.*, 1988; Fink-Puches & Smolle, 1993). NK cells are present in cervical lesions but in limited numbers and often with a reduction in their natural cytotoxicity (Tay *et al.*, 1987; Malejczyk *et al.*, 1989; 1993).

Macrophages may also play an important role in the control of HPV infection since, murine fibroblasts transfected with the early region or the E7 gene of HPV-16 are susceptible to being killed by activated macrophages (Denis *et al.*, 1989; Banks *et al.*, 1991). There is evidence of natural immunity to HPV infections from HPV lesions that manifest themselves as warts and lesions during immunosuppression. In humans, warts are prevalent and more likely to increase in size during conditions that depress T-lymphocyte functions, but these disappear once the immunosuppression is reduced or eliminated.

1.5.5 Cytokines associated with human papillomavirus infection

There is evidence that HPV infection may be under some sort of autocrine control of locally released cytokines especially in the early stages of infection, when decreased expression of anti-HPV cytokines by infected cells may favour HPV replication and lesion growth. Cultured keratinocytes are known to produce a variety of cytokines including IL-1, IL-6, IL-8, granulocyte-macrophage colony stimulating factor (GM-CSF) and tumour necrosis factor-α (TNF-α) (Kirnbauer *et al.*, 1989; Luger & Schwarz, 1990; Barker *et al.*, 1991). However, cervical keratinocytes immortalised by HPV-16 or –18 show a significant reduction of these cytokines when compared to normal cells (Woodworth & Simpson, 1993). The epidermal growth factor (EGF) family may play an important role in the growth and differentiation of HPV associated lesions and cells (Bauknecht *et al.*, 1989; Goppinger *et al.*, 1989; Zyzak *et al.*, 1994). Up-regulation of the EGF-receptor (EGF-R) appears to be a common feature of different HPV-associated lesions and of HPV immortalised cell lines (Gullick *et al.*, 1986; Viac *et al.*, 1987;Vambutas *et al.*, 1993).

Conversely, cytokines with anti-HPV activities may originate from infiltrating leukocytes as well as other non-epithelial skin, mucosal cells or fibroblasts. Cytokines involved in anti-viral responses may include TNF-α and IL-1, both of which have been shown to down regulate E6/E7 mRNA expression in HPV-16 and HPV-18 transfected cell lines. TNF-α also inhibits the proliferation of other HPV containing cell lines (Malejczyk *et al.*, 1992; Villa *et al.*, 1992; Kyo *et al.*, 1994; Rösl *et al.*, 1994; Woodworth *et al.*, 1995). Transforming growth factor-β (TGF-β) also has inhibitory effects on HPV mRNA expression at the transcriptional level leading to

the loss of E6 and E7, possibly due to down-regulation of *c-myc* (Woodworth *et al.*, 1990; Braun *et al.*, 1990).

Both TNF-α and TGF-β have been detected in HPV associated lesions and are spontaneously released in culture supernatants of HPV-16 harbouring cell lines, although decreased TGF-β expression has also been reported (Woodworth *et al.*, 1990; Braun *et al.*, 1990; 1992; Malejczyk *et al.*, 1991; 1992; Ho *et al.*, 1994; Comerci *et al.*, 1996). Although IFN-γ may inhibit HPV-18 RNA expression and the proliferation of HPV containing cell lines, the functions of IFN-α remains unclear (Nawa *et al.*, 1990; Woodworth *et al.*, 1992; Kyo *et al.*, 1994). GM-CSF has been shown to aid recruitment of Langerhans' and dendritic cells into HPV transformed cervical epithelium and may be useful as a new immunotherapeutic approach for cervical cancer and SIL (Hubert *et al.*, 1999).

It is possible that HPVs may act to escape the immune system by reducing pro-inflammatory cytokine signals.

1.5.6 Mucosal immunity in the female reproductive tract

The female reproductive tract is largely protected by the mucosal immune system. Plasma cells in the lamina propria regions secrete either IgA_1 or IgA_2 antibodies in response to stimulus. The main effector site is the uterus with organised lymphoid tissue in the endocervix comparable to Peyers patches of the gastrointestinal tract (Hordness *et al.*, 1996; Jalanti & Isliker, 1977). Cells of the immune system drain through the cervical, rectal and gastric lymph nodes, all of which may cover reproductive tract immunity (Miller *et al.*, 1992).

Immunity may depend to some degree on the levels of steroid hormones, as IgG and IgA levels fluctuate during the menstrual cycle. Both IgG and IgA levels in cervical secretions are decreased during midcycle when cervical mucus is the thinnest, probably providing better penetration of sperm as well as micro-organisms which may contribute to increased sexually transmitted diseases in the second part of the menstrual cycle (Tristram & Ogra, 1994). Further evidence for hormonal control is the up-regulation in the uterus and down regulation in the vagina of secretory component and IgA following treatment with oestradiol, whereas treatment with progesterone results in down regulation of both secretory component and IgA (Wira *et al.*, 1995).

Antigen presentation also varies during the reproductive cycle and is lowest at oestrus and highest at proestrus as the expression of MHC antigens varies (Edelstam *et al.*, 1992). Glucocorticoid hormones have been shown to reduce the expression of MHC-I antigens on human genital squamous cancer cell lines (Von Knebel Doeberitz *et al.*, 1990). This may relate to the increased risk of cervical cancer during pregnancy.

1.5.7 Cutaneous immunity

The skin consists of an epidermis separated from the underlying dermis by the basement membrane. Both epidermis and dermis contain cell populations that play active roles in immune reactions. In the epidermis keratinocytes produce a number of cytokines (see section 1.5.5) that stimulate the chemotaxis and activation of immune cells. Keratinocytes are also able to express MHC-II after exposure to IFN-γ, which is similar to the induction of MHC-II on macrophages (Abbas *et al.*, 1994). Langerhans' cells function as professional antigen presenting cells, interacting with

CD4+ lymphocytes. Intraepidermal lymphocytes constitute only 2% of skin associated lymphocytes, the rest reside in the dermis and are predominantly CD8+ although the specificity or function of this population is not clear. Similar to other connective tissues, the dermis contains T-lymphocytes (CD4+ & CD8+) predominantly in a perivascular location and scattered macrophages. The major type of T-lymphocyte mediated immune response in the skin is delayed type hypersensitity (DTH). This is a reaction to soluble protein antigens or chemicals that can bind to and modify self-proteins, creating new antigenic determinants. Little is known about humoral immune responses in the skin, although it is known that secretory IgA is present. B-lymphocytes are rarely encountered and it is probable that IgA produced by B-lymphocytes in draining lymph nodes is transported back to the skin *via* the circulation.

1.5.8 Methods used to detect human papillomavirus specific immune responses

Before the heterogeneity of HPV types was appreciated, early studies employed virions derived from pooled plantar warts (Pfister, 1984). Although untyped, these virions were used to look for antibodies in patients with genital warts!

HPV-11 virions obtained from nude mice xenografts have also been used to examine HPV specific immune responses from subjects with CA and laryngeal papillomas (Bonnez *et al.*, 1991). However, the yield of HPV-11 virions is extremely low and other HPV types have not been readily propagated as discussed previously.

Antigen derived from viral proteins expressed in bacteria, or synthetically constructed peptides, have been useful in determining antibody and cell mediated responses to individual proteins (Galloway, 1992; Tindle & Frazer, 1994; Viscidi & Shah, 1992).

Although bacterially derived proteins have several advantages, including a reproducible supply of antigen from any HPV type, most are insoluble and have to be used under denaturing conditions in Western blot assays.

As it is possible to construct VLPs in recombinant baculovirus and vaccinia virus, these have been investigated as a source of L1 and/or L2 antigens (Hagensse *et al.*, 1993; Kirnbauer *et al.*, 1992; Rose *et al.*, 1993). It is also possible to use chimeric VLPs, which include an early protein component (Muller *et al.*, 1997).

Antigens to be used in this study were synthetically constructed peptides. Attempts to develop functional assays to measure CMI have had limited success, although high stimulation indices have been reported for some synthetically constructed HPV proteins (Tindle & Frazer, 1994; Viscidi & Shah, 1992; Luxton *et al.*, 1996; Shepherd *et al.*, 1997).

1.6 TREATMENT OF HUMAN PAPILLOMAVIRUS ASSOCIATED LESIONS WITH VACCINES

Due to the viral aetiology of SIL and cervical cancer they may be prevented or treated using vaccines, as are other viruses such as poliovirus and rabies virus (Brochier *et al.*, 1994).

Three well documented HPV clinical trials are currently underway: (i) Holland, this consists of two high affinity HLA-A0201 binding peptides from HPV-16 E7, together with a synthetic helper peptide and the Montamide ISA51 adjuvant. This is being tested on patients with HPV-16 associated tumours who have not responded to conventional therapy and who have a HLA-A0201 tissue type. (ii) Australia, A phase I/II trial has been initiated in patients with advanced cervical carcinoma. (iii) Wales,

the most promising trial to date involves vaccination with a recombinant vaccinia virus expressing HPV-16 and -18 E6 and E7 proteins. For safety reasons, portions of the E7 proteins have been mutated to inactivate the pRB binding site. All patients in this study responded with an antibody response to vaccinia virus. In addition, two patients who already had antibodies to E6 exhibited an increased titre while two others developed new antibody responses to E7. Two of four patients developed MHC-I restricted CTL responses but four patients who were HLA-A0201 positive did not show a CTL response when tested with target peptides of the Dutch trial.

There are two HPV vaccine strategies. Prophylactic vaccination with L1 and L2 to prevent viral infection and therapeutic vaccination with E1, E2, E4, E5, E6 and E7 to treat established lesions. A combination of the two may be valuable in treating HPV associated disease.

1.6.1 Prophylactic human papillomavirus vaccines

Considering VLPs appear identical to authentic HPV virions, it is feasible that they would make ideal antigens for prophylactic vaccination and also delivery vectors for a therapeutic component. They have already been investigated as immunogens against HIV and foxes have been successfully immunised using vaccinia virus and rabies virus recombinants (Barnes, 1987; Brochier *et al.*, 1994). The advantages of using VLPs are that: (i) their antigenic conformational structure displays a high degree of sequence conservation; (ii) immunisation with PV VLPs has shown protection against experimental BPV and CRPV infection by the production of neutralising antibodies (Breitburd *et al.*, 1995; Donnelly *et al.*, 1996); (iii) they lack potentially oncogenic

viral DNA; (iv) VLPs from different viral strains would provide a multivalent vaccine covering common HPV types.

Exposure of cells to VLPs *in vitro* leads to binding, uptake and transport of capsid proteins which are detected in the Golgi, suggesting that processing occurs through the MHC-I pathway (Schiller & Roden, 1995; Zhou *et al.*, 1995). This is also true for chimeric VLPs (L1 & E7) which induce neutralising antibodies that may provide both prophylactic and therapeutic immunity (Müller *et al.*, 1997). As investigations of VLPs in human volunteers continue, there are potential problems associated with the testing and introduction of prophylactic vaccines. Whilst testing their efficiency against HPV-16 infection is relatively simple, it may take several years to assess their efficacy against SIL and impossible to test their protective effects against cervical carcinoma for ethical reasons given the evidence for the oncology of cervical cancer associated HPVs. Cells in the genital tract expressing Fc receptors could theoretically take up antibody-coated HPV virions and antibodies may act as 'enhancing antibodies' and promote, rather than negate infection. HPV-16 variants occur with coding changes in the L1 protein (Crawford, 1993) and such variants along with the other genital HPVs may escape neutralisation mediated by the induction of antibodies to a single HPV-16 isolate. In advanced SIL and cervical cancers, when capsid proteins are no longer expressed such vaccines will cease to have any effect. Finally methods to develop VLPs need to be revised for large-scale production and consistency between batches.

1.6.2 Therapeutic vaccines to prevent viral replication

A complementary approach to prophylactic vaccines is the concept of vaccines able to induce the destruction of established HPV-16 containing lesions. Such an approach is feasible, as in mice CTLs raised against Adenovirus S viral nuclear oncogene E1A, were able to eradicate large established tumours of Adenovirus S transformed tumour cells (Kast *et al.*, 1991). Similarly, recombinant vaccinia virus containing the middle- and large-tumour (large-T) antigen of polio virus confers immunity to rats against tumours containing the full length viral genome (Lathe *et al.*, 1987). Indeed, much effort has been devoted to developing HPV-16 and -18 E6 and E7 based vaccines as these probably represent tumour specific antigens for the vast majority of HPV associated cancers, and as such, rational targets for the design of therapeutic vaccines. However, E6 and E7 are potentially oncogenic and so may not be ideal vaccine candidates, unless mutated or modified in some way. Protective CTL epitopes may be uncoupled from regions involved in binding to p53 and pRB. Truncated or mutated proteins could also be constructed. However, it is not known whether such mutations would interfere with the immunogenicity of either protein, but since CTLs recognise short peptides, most CTL epitopes are likely to be unaffected. Other early protein targets such as E1, E2 or E5 may be of potential benefit. They are expressed in low grade SIL, which contain HPV-16 DNA and high levels of E5 mRNA are detected in L-SIL (Stoler *et al.*, 1992). HPV-16 E5, although an oncogene, has only a weak transforming activity and so may be safer than using HPV-16 E6 or E7. It is known to remain expressed until very late stages of malignancy (Matsakura *et al.*, 1989; Durst *et al.*, 1992). The use of HPV-16 E5 may also be more appealing to the

pharmaceutical companies as it involves a potentially huge market for preventing the occurrence of SIL.

Considering that T-lymphocyte responses are MHC-restricted, a vaccine for use in outbred populations such as man would theoretically need to incorporate several HPV 16 CTL epitopes in order to induce a wide coverage of immunity. In most ethnic populations, at least 90% will express one of the six most common MHC alleles of that community (Tindle & Frazer, 1994).

1.6.3 Vaccine vectors

Vaccines designed to stimulate CTLs may be efficiently delivered *via* attenuated intracellular pathogens such as recombinant Bacille Calmette Guerian (BCG) or vaccinia virus incorporating HPV-16 CTL epitopes (Klein, 1990; Tartaglia *et al.*, 1992). BCG is a safe, well-tested vaccine that can be utilised as a vector to induce both strong humoral and cell-mediated immunity to foreign epitopes (Aldovini & Young, 1991; Stover *et al.*, 1991). HPV-16/BCG recombinants may prove to be particularly useful vectors, bearing in mind the vertical transmission of HPV-16 and -18. Such a vaccine could be given at 6 months of age by an oral route conferring long lived immune responses with a single dose.

Other suggested vectors for delivery of HPV 16 antigens include commensal organisms of the female genital tract for example Lactobacillus (Crawford, 1993). These could be used to induce HPV-16 specific CTLs, to destroy HPV-16 containing lesions. Similarly, recombinant *Salmonella typhimurium* may also serve as a suitable vector to induce mucosal antibodies and HPV-16 E7 has already been expressed in this system (Chatfield *et al.*, 1992).

The production of DTH in response to recombinant vaccinia virus/HPV-16 E7 has been reported in a mouse model (McLean *et al.*, 1993) It is thought that HPV may evade eradication by dampening the DTH effector response as a result of presenting viral antigen in very low concentrations (McLean *et al.*, 1993). By presenting high concentrations of HPV-16 E7 antigen, DTH responses are induced in mice and may represent effective mechanisms to combat HPV infections (Chambers *et al.*, 1994). Murine CTL responses to vaccinia virus expressed HPV-16 L1 protein have been defined (Zhou *et al.*, 1991) and immunisation which results in the eradication of strong DTH may be a potent mechanism for preventing persistent HPV infection. However, care must be taken in the selection of live vaccinia vectors since their use resulted in four deaths among (HIV) immunosuppressed patients (Culliton, 1992). To overcome this shortfall, highly attenuated strains of vaccinia virus such as the NYVAC-strain or other pox viruses such as the Canary pox (Lancet editorial, 1992) should be used instead.

Vaccines in live attenuated viral vectors are particularly useful, as protection can be spread by infection to individuals who have not attended for vaccination. Such attenuated vectors may also include poliovirus, which induces strong mucosal immunity characterised by secretory IgA antibodies and CMI at the site of infection. Indeed, a B-lymphocyte epitope of a HPV-16 L1/poliovirus chimera was immunogenic in rabbits (Jenkins *et al.*, 1990). Similarly, the equivalent epitope on BPV-2 L1 was also immunogenic but did not induce antibodies with neutralising activity for BPV-2 *in vitro* (Cason *et al.*, 1993).

Viral proteins and peptides are usually poor inducers of CTLs but the use of immunostimulatory complexes (ISCOMS) may permit the induction of CTLs to

administered proteins or synthetic peptides (Randall & Souberbielle, 1990). ISCOMs or liposomes are vesicles formed from synthetic lipid bilayer membranes composed of saponin and cholesterol. The production of ISCOMs can be modified to induce the formation of vesicles containing antigen which may potentiate cellular immune responses. Indeed, recombinant HPV-16 proteins within ISCOMs are believed to potentiate antigenicity (Jones *et al.*, 1988). Other delivery systems could also be used such as pulsed release of antigens from biodegradable microspheres (Eldridge *et al.*, 1989; Moldoveanu *et al.*, 1989) and plant virus based chimeras such as cowpea and tobacco mosaic virus: the latter has been used to produce potential anti-malarial vaccines (Turpen, 1995).

Other vectors include spleen dendritic cells, an *Escherichia coli* protein carrier ISCAR and Hepatitis B core antigen. These have protected against challenge with E6 or E7 transfected tumour cells and produced IgG and IgG2α antibodies as well as IL-2 and IL-4 (Tindle *et al.*, 1995; Ossevoort *et al.*, 1995).

1.7 *PRIORI* RATIONALE

Reasons for this investigation arise from the fact that HPV-16 E5, a minor oncogene, is largely ignored in the context of HPV immune intervention whilst attention has primarily focused on E6 and E7, the major oncogenes of HPV-16. It was postulated that immune responses specific for HPV-16 E5 protein may occur as:

(i) The HPV-16 E5 gene is expressed soon after infection, with both mRNA and protein detectable in low-grade SIL (Stoler *et al.*, 1992; Kell *et al.*, 1994; Biswas *et al.*, 1997). The prevalence of HPV-16 E5 mRNA increases with advancing severity of disease (Biswas *et al.*, 1997), suggesting that the contribution of HPV-16 E5 protein to tumour development occurs in the early stages of infection when HPV DNA is still episomal. There is also evidence to indicate HPV-16 E5 expression persists from low grade lesions through to cervical cancer in integrated and episomal forms as mRNA transcripts have been demonstrated in cervical cancers (Matsukara *et al.*, 1989; Durst *et al.*, 1992; Cavuslu *et al.*, 1996b).

(ii) HPV-16 E5 protein is probably expressed at the cell surface as a result of endosome recycling of ligands and formation of inter-cellular gap junctions (Goldstein *et al.*, 1985; Oelze *et al.*, 1995), making it available to cells of the immune system.

(iii) Finally, the amino acid sequences of HPV E5 proteins are poorly conserved between different types making them potentially type specific targets and thus any data obtained in this study will be specific for HPV-16 only.

Although, the immunogenicity of HPV-16 E5 has not been extensively studied, antibodies to E5 synthetic peptides have been reported in humans (Dillner, 1992). Furthermore, antibodies to HPV-16 E5 have been successfully raised in New Zealand

white rabbits and the resulting anitbodies recognised E5 protein in cervical lesions (Kell *et al.*, 1994). Prior to this thesis, there have been no reported studies investigating CMI to HPV-16 E5.

1.8 AIMS AND OUTLINE OF THESIS

The initial aim of work outlined in this thesis was to characterise HPV-16 E5 specific immune responses in women with and without HPV-16 infection. In *Chapter Two,* it was anticipated that cell mediated immunity would be inversely correlated with disease severity. To investigate this, peripheral blood mononuclear cells were isolated from each subject and stimulated with HPV-16 E5 peptides. Cell mediated immune responses were recorded as stimulation indices. As the HPV-16 E5 ORF is subject to DNA variation, in *Chapter Three,* the presence of HPV-16 E5 variants from DNA amplified from study subjects was investigated. The current school of thought applied to HPV serology implies that the presence of HPV specific antibodies may be associated with severity of lesions. To test this hypothesis, reference and variant E5 peptides were investigated for serum reactivity. *Chapter Four-* Immune responses specific for HPV-16 E5 peptides were investigated after immunisation of Balb/c mice. Also covered within this chapter is an investigation of the ability of E5 peptides used in immunisation for binding to MHC-I antigens. *Chapter Five-* From these studies described above, it was determined that E5 was a poor immunogen, therefore, both E5 peptides and DNA were studied for effects on MHC-I expression and for effects on intracellular transport. *Chapter Six-* The data of the whole thesis are discussed.

Chapter Two

Characterisation of Human Papillomavirus Type-16 Infection and Cell Mediated Immune Responses in Women with or without Cervical Disease

2.1 INTRODUCTION

As there is evidence to suggest that CMI is important in controlling HPV infection, it is probable that cervical disease occurs as a result of a defective immune response. For example, in L-SIL and H-SIL, there are reduced numbers of the antigen presenting Langerhans' cells, those that are present exhibit changes in morphology (Hughes *et al.*, 1988). There are also reductions in the number of intraepithelial T-lymphocyte numbers in all grades of SIL (Morris *et al.*, 1983). In contrast, increased numbers of MHC-I restricted CD8+ T-lymphocytes are present locally in cervical cancer (Ghosh *et al.*, 1994) but the implication of this finding remains unclear.

Proliferative peripheral blood mononuclear cell (PBMC) responses to HPV-16 E6 and HPV-16 E7 Th epitopes have been detected in healthy volunteers (Amtmann *et al.*, 1992). Indeed, proliferative lymphocyte reponses to an E7 peptide were related to ongoing infection with HPV-16 (Kadish *et al.*, 1994). As the E6 and E7 proteins of HPV-16 are persistently expressed throughout infection and malignancy, most assays of proliferative T-lymphocyte responses to HPV epitopes have focused primarily on these (Luxton *et al.*, 1997). However, as previously discussed in section 1.6, E6 and E7 may not be the sole targets for immune intervention as other proteins encoded by the early ORFs (E1, E2, E4 and E5) may also contain major T-lymphocyte epitopes.

The aims of this chapter were to investigate CMI to E5 in relation to infection with HPV-16 and with severity of HPV-16 associated cervical disease. As discussed in section 1.7, the protein encoded by the HPV-16 E5 ORF is associated with cellular membranes and may be expressed at the cell surface (Figure 2.1) where recognition by cells of the immune system may occur. If this is the case, then it is anticipated that subjects without cervical disease or those with L-SIL are more likely to exhibit HPV-16 E5 specific proliferation as this is the stage of HPV-16 associated disease when E5

transcripts are abundant.

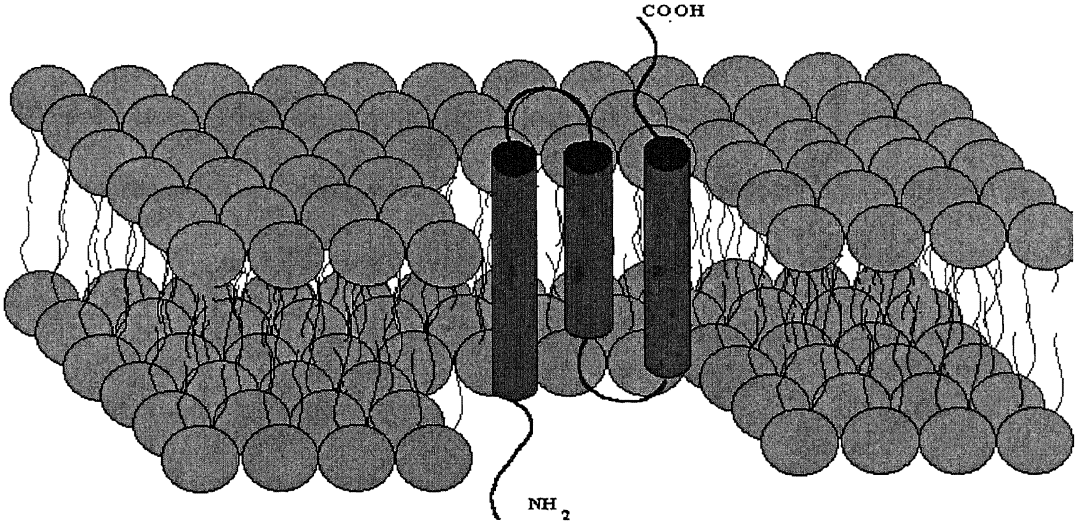


Figure 2.1 Predicted conformational structure of human papillomavirus type-16 E5 protein in a cellular membrane. The above figure demonstrates the predicted conformational structure of HPV-16 E5 in a cellular membrane. The location of HPV-16 E5 within the cell membrane implies that it interacts with a completely different set of cellular factors to those interacting with the E6 and E7 nuclear oncogenes. A role in the disruption of the normal signalling processes from a family of growth factors has been suggested (Banks & Matlashewski, 1996). The SOUSI database predicts HPV-16 E5 amino acid sequence as a membrane protein with three transmembrane helices. The DAS transmembrane prediction server also predicted three transmembrane helices with no N-terminal signal sequences, mitochondrial or nuclear localisation signals. **COOH** carboxyl terminus, **NH_3** amino terminus (Nakai & Kanehisa, 1991).

Subjects were tested for HPV-16 DNA using primers for both the E5 and the E6 ORFs. The detection of HPV-16 E6 was included as along with E7, it is a major transcript of HPV-16 DNA, providing more opportunity for the detection of HPV-16 DNA, *i.e.*

positive identification of HPV-16 positive patients.

A reverse transcription-PCR (RT-PCR) assay was performed to detect HPV-16 E5 messenger RNA (mRNA). This enabled the detection of active HPV-16 infections producing HPV-16 E5 protein.

Peripheral blood mononuclear cells (PBMCs) were isolated and tested for proliferative responses to HPV-16 E5 synthetic peptides. Proliferative PBMC responses were characterised according to HPV-16 infection and to disease severity.

The antigens used in this chapter were synthetic peptides constructed by solid phase peptide synthesis (SPPS). Antigens constructed using SPPS have been used in many investigations of HPV specific immune responses (Jocmus-Kudielka *et al.*, 1989; Mann *et al.*, 1990; Mandelson *et al.*, 1992; Muller *et al.*, 1992; Hamsikova *et al.*, 1994; Sun *et al.*, 1994). Similarly, synthetic peptides have also been used in the viral diagnosis of other virus types including hepatitis C virus, respiratory syncytial virus and the Japanese encephalitis virus (Poisson *et al.*, 1993; Sharma *et al.*, 1996; Langedijk *et al.*, 1996; Huang *et al.*, 1996). For stimulation of PBMCs 9-mer peptides corresponding to whole length HPV-16 E5 when overlapped by 4 amino acids were used (Figure 2.2). This length of antigen was constructed for two reasons. Firstly, as HPV-16 E5 is extremely hydrophobic it would be difficult to solubilise in cell culture media for stimulation of PBMCs. Secondly, this length of peptide will bind to both antigen binding grooves of MHC-I and MHC-II molecules whilst allowing for a degree of epitope mapping.

2.2 METHODS

2.2.1 Subjects

Women were recruited from 'well-woman' and outpatient clinics at St Thomas' Hospital (London, U.K.). Fifty five women had recently undergone histological diagnosis to

confirm abnormal cervical smear results, twenty women with normal cervical smear results were also recruited (Table 2.1). Clinical samples were collected by a gynaecologist, once ethical permission had been granted (The Research Ethics Committee of St Thomas' Hospital).

Table 2.1 Characteristics of study subjects

	Total	Normal Cytology	Cervical Disease	L-SIL	H-SIL	Cervical Cancer
	75	20/75	55/75	15/75	33/75	7/75
Mean age (years)	30	35	28	28	28.5	61
Age range (years)	18-83	18-79	19-83	19-53	20-45	24-83

Patients were not selected on an age-matched basis but the age range of asymptomatic women and patients with cervical disease was not significantly different. When patients were divided into grade of disease, cervical cancer patients were significantly older compared to asymptomatic women (ut: $p=0.03$) but not when compared to patients with H-SIL or L-SIL (both $p>0.05$). Indeed, cellular changes associated with HPV infection are thought to occur more frequently in younger women (Kainz *et al.*, 1995).

2.2.2 Sample collection

Cervical brush smears were collected with Axibrushes™ (Colgate Medical Ltd.), into 5 ml of sterile phosphate buffered saline (PBS), consisting of 154 mM sodium chloride; 8.1 mM mono-potassium phosphate; 1.9 mM disodium hydrogen orthophosphate, pH 7;

(BDH Ltd., U.K.) in sterile universal containers (Sterilin Ltd., U.K.). Four ml were stored in 1 ml aliquots at -70°C in cryovials for DNA PCR (Costar Corning Ltd., U.K.). The fifth aliquot was centrifuged at room temperature and the cell pellet re-suspended in 400 µl of RNAzol B (Biotex Inc.). This was also stored at -70°C until required for RT-PCR. Four ml of peripheral blood were collected into tubes containing no added anticoagulant from which serum was prepared by centrifugation at 500 g for 10 min at room temperature and stored at -20°C (see section 3.1.1). Sixteen ml of peripheral blood were taken into tubes containing 0.02 g ethylene diamine tetra-acetic acid; disodium salt (EDTA; BDH) and PBMCs were isolated by layering whole blood onto 24 ml of Ficoll-Paque™ (Pharmacia Biotech U.K.) and centrifugation at 300 g for 15 min at 4°C. Cells at the interface were harvested, washed three times by re-suspension in, then centrifugation through, Roswell Park Memorial Institute medium-1640 (RPMI 1640; Sigma-Aldrich Company Ltd., U.K.). RPMI-1640 was supplemented with 10% (volume for volume; v/v) foetal bovine serum (FBS; Gibco Ltd., U.K.) 2 mM l-glutamine (Sigma), 100,000 units/L benzylpenicillin and 0.1 g/L streptomycin sulphate (Sigma). Cells were re-suspended in 10% (v/v) dimethyl sulphoxide (Sigma) in FBS on ice, divided into three aliquots and frozen at -1°C/min to -70°C in 1.8 ml cryotubes (Costar) using a freezing chamber (Nalgene) and transferred to liquid nitrogen. All reagents used in this thesis were of Analar grade unless otherwise stated.

2.2.3 Polymerase chain reaction assays

When required, a 1 ml aliquot of frozen cervical cells was thawed and transferred into microcentrifuge tubes (Sterilin). Samples were vortexed for 10 s to re-suspend cellular material and then centrifuged at 10,000 g for 10 min at room temperature to pellet cellular material. Erythrocytes, if present (assessed by visual inspection), were lysed by

washing cell pellets in 1 ml Tris-EDTA buffer (10 mM Tris-hydrochloride; 1 mM EDTA; BDH). Cell pellets were re-suspended in 200 µl of Proteinase K reaction buffer consisting of 50 mM potassium chloride, 2.5 mM magnesium chloride, 10 mM Tris-hydrochloride pH 8.3, 0.45% (v/v) NP40™, 0.045% (v/v) Tween 20™ (Sigma) and 0.06 g/L (15 units/mg) Proteinase K; (Boehringer Mannheim U.K.). They were then incubated overnight at 55°C followed by an incubation at 95°C for 10 min to inactivate the proteinase K. Samples were not subjected to a phenol/chloroform DNA extraction as there is a variable loss of target DNA in these samples which can result in false negative results (Mant *et al.*, 1997).

To reduce the non-specific amplification of irrelevant DNA species all PCRs were performed using a 'hot start' method, in which the sample is separated from the oligonucleotide primers by a wax plug (D'Aquila *et al.*, 1991; Chou *et al.*, 1992). In a clean 'amplicon free' laboratory, PCR reaction mixtures were prepared as two 'master mixes', an upper and a lower phase (Table 2.2). The lower solution was mixed in a 0.6 ml volume microcentrifuge tube, two wax pellets (Fibrowax: BDH) were placed above the mix and heated to 70°C for 2 min allowing the wax to melt and seal in the lower mix. The wax was allowed to solidify at room temperature for 2 min after which the upper phase was added.

Tubes were taken to a sample preparation laboratory where 10 µl of sample were added to the upper phase and then to a third laboratory where they were placed in a thermal cycler utilising a thermocouple to monitor the internal tube temperature (Hybaid Thermal Reactor; Hybaid Ltd, UK). Samples were then subjected to the appropriate cycles of denaturation, primer annealing and elongation programmed (Table 2.3). To control for quality of the samples with respect to non-specific inhibitors of PCR and the quantity of DNA obtained, clinical samples were tested for human genomic β-globin

DNA in a PCR using primers PC04 and GH20 (Table 2.4).

Only those samples positive for β-globin DNA were considered suitable for HPV-16 DNA PCR analyses. Samples were screened for HPV-16 DNA using primers for HPV-16 E6 and HPV-16 E5 DNA (see Table 2.4).

Controls were included for every 10 samples tested; the positive control was the HPV-16 genome contained within the *Pat16* plasmid (Jewers *et al.*, 1992) and the negative control contained DNA extracted from the control HPV negative A431 cell line (European collection of cell cultures, ECACC.).

After amplification, 18 µl of PCR product were mixed with 2 µl of DNA loading buffer consisting of 30% Ficoll (weight for volume; w/v); 250 mM EDTA (Sigma) and 0.4% Orange-G (w/v; BDH). Samples were visualised after separation by electrophoresis through a 2% agarose gel. The gel was prepared by heating agarose (Sigma) in Tris/borate/EDTA buffer (TBE), consisting of, 0.9 M Tris-hydrochloride, 0.9 M boric acid (BDH) and 0.002 M EDTA to 97°C for 2 min. This was allowed to cool to 50°C and 0.05 g/L of ethidium bromide (Sigma) were added per 100 ml agarose gel which was permitted to solidify in a flat bed electrophoresis tank (Hybaid). Samples were loaded into wells of an agarose gel and an electrical field of 75 volts, 45 milli amps was applied for 45 min at room temperature. A 1,000 bp molecular weight ladder (Life Technologies Ltd., UK) was separated in parallel with amplified DNA. After electrophoresis, gels were transilluminated with ultraviolet light and photographed using the Appligene Imager System (Figure 2.2 a,b). Subjects were considered positive for HPV -16 DNA if positive for either E6 or E5 DNA in PCRs as determined by bands of the required length.

Table 2.2 Volumes for single PCR reaction mixes

	Reaction Volumes		
Reagents	**HPV-16 E5** **HPV-16 E6**	**Nested HPV-16 E5** **β-Globin**	**Final** **Concentration**
Lower phase 35 µl			
10 × PCR Buffer	5 µl	5 µl	25 mM KCl; 5 mM Tris-HCl, pH 9; 0.05% Triton X-100 (Promega)
$MgCl_2$	10 µl	10 µl	2.5 mM
DNTPs	8 µl	8 µl	0.8 mM (0.2 mM each)
Primer U	1 µl	1 µl	0.5 µM
Primer D	1 µl	1 µl	0.5 µM
H_2O	10 µl	10 µl	——
Upper phase 65 µl			
10 × PCR Buffer	5 µl	5 µl	25 mM KCl; 5 mM Tris-HCl, pH 9; 0.05% Triton X-100 (Promega)
T.*aq*	0.5 µl	0.5 µl	25 units/ml
MH_2O	49.5 µl	55.5 µl	——
DNA Template	10 µl	4 µl	——

Volumes in microlitres are quantities necessary for one 100µl PCR reaction when preparing 'master mixes' figures are multiplied by $n \times 1.1$ (where n = the number of reactions to be performed) to allow for wetting of plastics. ***T.aq.***, *Thermus aquaticus* DNA polymerase; **dNTPs.**, precursor deoxynucleoside triphosphates; **U.**, upstream primer; **D.**, downstream primer. **KCl** potassium chloride, **HCl** hydrochloride, **H_20** molecular biology grade distilled water, **$MgCl_2$** magnesium chloride.

Table 2.3 PCR cycling conditions

Number of Cycles	β-Globin	HPV-16 E5	HPV-16 E6
1	94°C 5 min	94°C 5 min	94°C 5 min
40	95°C 30 s 53°C 30 s 72°C 1 min	94°C 15 s 55°C 15 s 72°C 5 min	94°C 1 min 55°C 1 min 72°C 1 min
1	72°C 5 min	72°C 5 min	72°C 10 min

These cycling conditions were optimised for the Hybaid thermocycler and designed for high sensitivity. The thermocycler was programmed to bring the heating block up to temperature within 1 second.

Table 2.4 Oligonucleotide primers

Protein/primer name	Oligonucleotide Sequence	position	Size (bp)	Ref
β-globin GH 20 PC 04	$^{5'}$GAA GAG CCA AGG ACA GGT AC$^{3'}$ $^{5'}$CAA CTT CAT CCA CGT TCA CC$^{3'}$		286	I
Human Keratin HK-1 HK-2	$^{5'}$GAG GCA GCG GCC TGC TGA G$^{3'}$ $^{5'}$GGC CAG ACG GGC ATT GTC AA$^{3'}$		220	II
HPV-16 E6 C-16-E6-D C-16-E6-U	$^{5'}$TTG GAT CCG AGA ACT GCA ATG TTT CAG GAC$^{3'}$ $^{5'}$ACA GAT CTA TTA CAG CTG GGT TTC CTA CG3'	94-113 562-543	482	III
HPV-16 E5 C-16-E5-U C-16-E5-D	$^{5'}$ATT TAG ATC TAT ATG ACA AAT CTT GAT ACT GC3' $^{5'}$TAC AGG ATC CTT ATG TAA TTA AAA AGC GTG CAT$^{3'}$	3837-3868 4110-4078	482	III
HPV-16 E5 RT-PCR P1 P2 P3 P4	$^{5'}$TTT TTT TTT TTT TTT TTT TTT TTT TTA AGT$^{3'}$ $^{5'}$ATT TAG ATC TAT ATG ACA AAT CTT GAT ACT GC$^{3'}$* $^{5'}$TAC AGC ATC CTT ATG TAA TTA AAA AGC GTG CAT$^{3'}$* $^{5'}$GTG CTT TTG TGT GTC TGC CTA TTA ATA C$^{3'}$	4227-4330 3837-3868 4078-4110 3910-3936	421 202	III, IV & V

bp: base pairs; **HPV**: human papillomavirus; **A**: adenine; **T**: thymine; **G**: guanine; **C**: cytosine; **position** relates to the nucleotide position on the HPV-16 reference isolate. **Ref**: Reference (**I**) Saiki *et al.*, 1986; (**II**) Bosma *et al.*, 1995; (**III**) Cavuslu *et al.*, 1996; (**IV**) Biswas *et al.*, 1997; (**V**) *Schwartz *et al*, 1985. All primers were made by Oswel DNA services Ltd., U.K.

2.2.4 Reverse-transcription polymerase chain reaction assays

The aliquot of pelleted cervical cells stored in RNAzol B was thawed and washed once with 70% (v/v) aqueous ethanol (Sigma), air dried and re-suspended in 50 µl of Tris EDTA buffer (2.2.3) in a microcentrifuge tube.

Samples were incubated overnight at 37°C with 5 U of DNase I (Amersham Life Sciences Ltd., U.K.) in 10 µl of 100 mM magnesium chloride containing 10 mM dithiothreitol (Sigma) and 3.3 U of the RNase inhibitor guanidium thiocyanate (InhibitAce; 5'→3' Inc). An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v/v, Sigma) was added to the RNA pellet, vortexed for 30 seconds then centrifuged for 5 min at 13,000 g (Sambrook *et al.*, 1989). The upper phase (100 µl) was transferred to a fresh microcentrifuge tube and an equal volume of ethanol was added to precipitate the RNA. 75 µg of linear polyacrylamide (Life Technologies) was also added to the sample as a carrier to facilitate visualisation of the precipitated RNA (Gaillard & Strauss, 1990).

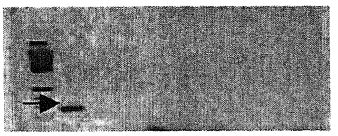
RNA pellets were re-suspended in 20 µl of RNase free molecular biology grade distilled water (H_2O). One negative control containing H_2O was processed along side every 5 samples. Reverse transcription was primed by 2.5 micro molar (µM) of a 25-30 dT oligonucleotide (dT; Pharmacia) with Moloney murine leukaemia virus reverse transcriptase (Life Technologies). The final volume of 20 µl contained 200 U of reverse transcriptase, 0.7 U of InhibitAce, 1 mM of each deoxynucleoside triphosphate, 5 µl of 5 times (×) first-strand buffer (Life Technologies) and 3 µl of RNA sample. Assays were performed with and without reverse transcriptase to confirm that mRNA and not contaminating DNA was detected. Samples were incubated at 37°C for 90 min and then at 99°C for 5 min to inactivate the reverse transcriptase. Primers P1 and P2 (Table 2.4)

were used in first round hot start PCR to produce a 421 bp amplicon (Figure 2.2 c). The 100 µl PCR mixture included the 20 µl reverse transcription mixture, 2.5 U of *Thermophilus aquaticus* (*T.aq*) DNA polymerase (Promega Corporation U.K.) in a final concentration of 4 mM magnesium chloride, 0.5 µM P1 and 0.5 µM P2.

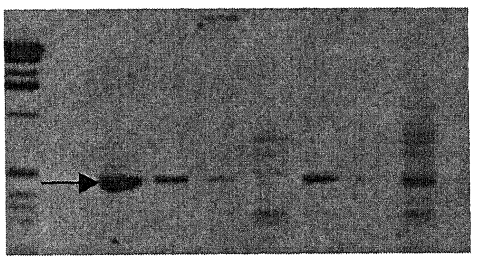
Samples were placed on a thermocycler (Hybaid) and incubated at 94°C for 5 min (1 cycle) followed by an incubation at 94°C for 5 min 49°C for 15 s, 72°C for 10 s (25 cycles) and a final incubation of 72°C for 5 min.

Reactions that produced amplicons of 421 bp after electrophoresis through a 2% agarose gel (Figure 2.2 c) were subjected to a further nested PCR employing primers P3 and P4 (Table 2.4; Biswas *et al.*, 1997) to produce amplicons of 202 base pairs. The nested PCR mix included 10 µl of the first round PCR product, 2.5 mM magnesium chloride 0.5 µM of P3, 0.5 µM of P4 and 2.5 U of *T.aq*. The PCR mixes were placed on a thermocycler and incubated 94°C for 5 min (1 cycle), 94°C for 15 s, 50°C for 15 s, 72°C for 10 s (25 cycles) followed by a final incubation of 72°C for 5 min. A nested RT-PCR for human keratin mRNA (Table 2.4; Bosma *et al.*, 1995) was performed in parallel to EmRNA on all samples to ensure that clinical samples were suitable for amplification by RT-PCR.

a



b



c

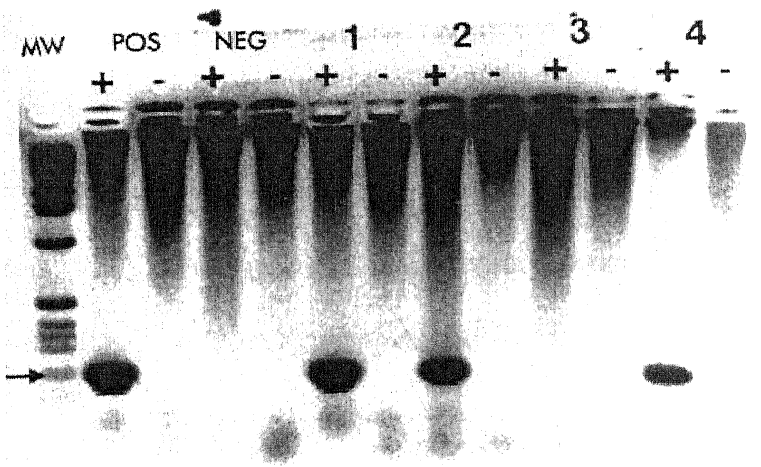


Figure 2.2 Photographs representative of PCR products after electrophoresis through a 2% agarose gel stained with ethidium bromide

a. HPV-16 E5, **b.** HPV-16 E6 **c.** HPV-16 E5 RT-PCR using P3 and P4 primer pairs **MW** refers to molecular weight marker, **pos** positive control, **neg** negative control, + addition of reverse transcriptase, - absence of reverse transcriptase. Arrow indicates a positive band at **a** 273 bp, **b** 482 bp, **c** 202 bp.

2.2.5 Synthetic peptides

Solid phase peptide synthesis (SPPS) is based on the sequential addition of alpha (α) amino and side chain protected amino acid residues to an insoluble polymeric resin support (Houghton *et al.*, 1985). After removal of the protective group, the next protected amino acid derivative is added. The resulting peptide is attached to the resin, *via* a linker, through its C-terminus and may be cleaved to yield a peptide amide. Side chain protecting groups are designed to be cleaved simultaneously with detachment of the peptide from the resin.

Synthetic peptides were synthesised from the carboxylic terminus stepwise toward the amino terminus and relied upon the base labile Fmoc (9-Fluorenylmethyloxycarbonyl) - group for N-α-protection amino acids. Cleavage of this protecting group was achieved by piperidine (Sigma) and final cleavage of the peptide resin and side chain deprotection by the strong acid trifluoroacetic acid (TFA, Sigma). N,N-dimethylformamide (DMF; BDH) was the primary diluent/solvent used for resin deprotection, coupling and washing. Peptide synthesis was carried out in a continuous flow manner, where the inert 4-(α-Fmoc-amino-2',4'-dimethoxybenzyl) phenoxyacetic acid resin (UltraSyn-C; Novabiochem, U.K.) was contained in a column through which reagents and solvents were pumped continuously under semi-automatic control (Biocycle; Pharmacia, U.K.). The UltraSyn-C resin has an acid labile 'handle' specifically designed for the synthesis of peptide amides by the Fmoc polyamide method. The use of this synthesis system allows easy removal of excess reactants and by-products with continuous solvent flow through the column. All reactions were performed in a fume cabinet.

For each peptide, 0.25 g resin was pre-swollen in DMF in a glass beaker and after removal of fine factory dust, were transferred to the peptide synthesis reaction column. The column was placed in the Biocycle and resin washed with DMF for 5 min. After

washing, resin was deprotected with 20% (v/v) piperidine in DMF (Millipore, U.K.) for 10 min. Resin was washed again with DMF for 5 min, to remove excess piperidine. 0.5 mM of the relevant amino acid Pfp ester (Novabiochem) and 0.5 mM (0.067 g) 1-hydroxybenzotriazole (HOBt; Sigma) were then dissolved in DMF and loaded onto the column. HOBt is included in the amino acid addition to prevent racemisation and dehydration of amino acids, as well as acting as a catalyst (Konig & Geiger, 1970). This mixture was allowed to re-circulate through the column for 45-60 min. The system was then flushed out with DMF for 5 min. Subsequent amino acids were added to the peptide chain in a series of cycles of deprotection, amino acid addition and DMF washing.

When the peptide of required length was constructed the amino (N)-terminal Fmoc group was removed with 20% (v/v) piperidine in DMF, prior to cleavage from the resin. Resin was placed in a glass universal and washed with DMF, acetic acid and then DMF again before being allowed to dry overnight. The cleavage cocktail consisting of, 94% (v/v) TFA, 5% (v/v) phenol and 1% (v/v) ethandithiol (BDH) was added to dried resin (Atherton & Shepherd, 1989; Fields & Nobel, 1990). Cleavage was performed at room temperature for at least 6 hours (h), with occasional swirling. Cleavage cocktail containing the cleaved peptide product was decanted off into a clean glass universal. Resin was washed twice with clean TFA and all washings were combined with the cleaved product. Peptide was precipitated by the addition of 8-10 volumes of cold diethyl ether (BDH). Washing by evaporation of ether with a nitrogen stream was performed and five ether washes carried out. After the final ether wash peptide was dissolved in 10% (v/v) aqueous acetic acid and then lyophilised using a Herto Freeze dryer (DK-345, Prior Labs.).

2.2.6 Short term peripheral blood mononuclear cell lines

One aliquot of frozen PBMCs was thawed, the viability assessed using trypan blue dye exclusion (see Appendix 1) and the cell number adjusted to 1×10^5 viable PBMCs/ml in stimulation media consisting of, RPMI 1640, 10%FBS (v/v), 2×10^4 units recombinant human IL-2 (Sigma), 4×10^3 units recombinant human IL-4 (Sigma) and an equimolar 2 mM (final concentration) mix of E5 HPV-16 9-mer peptides (Figure 2.3).

Whole length HPV-16 E5

^{1}MTNLDTASTTCCACFLLCFCVLLCVCLLIRPLLLSVSTYTSLIILVLLLWITAASAFRCFIVYIIFVYIPLFLIHTHATNQIY^{83}

9-mer peptides

^{1}MTNLDTAS^{8} ^{15}FLLCFCVLL^{23} ^{30}RPLLLSVST^{38} ^{45}LVLLLWITA^{53} ^{60}FIVYIIFVY^{68} ^{75}HTHATNQIY^{83}

^{5}DTASTTCCA^{13} ^{20}CVLLCVCLL^{28} ^{35}SVSTYTSLI^{43} ^{50}WITAASAFR^{58} ^{65}IFVYIPLFL^{73}

^{10}TCCACFLLC^{18} ^{25}VCLLIRPLL^{33} ^{40}TSLIILVLL^{48} ^{55}SAFRCFIVY^{63} ^{70}PLFLIHTHA^{78}

Figure 2.3 Synthetic HPV-16 E5 peptides. 9-mer peptides were designed with a 4 amino acid overlap to ensure that epitopes would not be lost due to the design of peptides. Amino acid positions are shown in superscript.

To each well of a 96 flat bottom well microtitre plate (Corning) 200 µl of PBMCs were dispensed and incubated at 37°C in a tissue culture incubator providing a humidified atmosphere containing 5% (v/v) carbon dioxide (CO_2) in air. After 72 h (day 3) the medium was changed and cells were incubated for a further 96 h (day 4) after which they were washed and re-suspended in fresh stimulation media.

A second aliquot of PBMCs was thawed and treated with 1 g/L mitomycin-C for 3 h at 37°C, washed twice in Dulbeccos Phoshate Buffered Saline (DPBS; Sigma) and re-suspended in RPMI 1640 media supplemented with 10% FBS. These cells were adjusted to 2×10^4 in a volume of 50 µl and added to each corresponding well as autologous antigen presenting cells. This method was first assessed in laboratory donors (see Appendix 4).

2.2.6.1 Proliferative responses of peripheral blood mononuclear cells to Human papillomavirus -16 E5 peptides

On day 14, cells were washed three times in DPBS (as above) and adjusted to 1×10^5 viable PBMCs/ml. Fifty µl volumes were dispensed into 18 to 21 wells of a 96-well flat-bottomed tissue culture plate (Corning). To three wells, 0.012 g/L (final concentration) concanavalin A (Sigma) were added, to ensure that cells were viable and capable of proliferation. Another three wells contained just RPMI 1640/FBS and the remaining wells contained 2 mM pooled 9-mer peptides (final concentration).

Forty-eight hours later (day 16), 50 µl of a solution containing 2 g/L of the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma; Denizot & Lang, 1986) in RPMI 1640/FBS were added to each well for 4 h at 37°C (1 g/L final concentration). Plates were centrifuged at 400 g for 5 min at room temperature,

the supernatant removed by aspiration and 100 µl/well of dimethyl sulphoxide were added aseptically to solubilise the purple precipitate from mitochondria. Colour development (absorbance at a wavelength of 570 nm; A_{570}) was assessed after a 10 min incubation at room temperature using a Labsystems Multiscan MCC 349 plate reader (see Appendix 2).

Results were expressed as stimulation indices (SI) representing the absorbance at A_{570} of cells stimulated by peptides divided by the A_{570} of cells which were not stimulated with HPV-16 E5 peptides. Subjects were considered to have positive responses if at least five wells had SI values in excess of 1.5 and at least one greater than 2.499: data are expressed as the mean of all wells greater than 2.499. Those cultures with SI values below 1.5 were considered negative.

2.2.6.2 Detection of interferon gamma in culture supernatants

The production of INF-γ; TNF-α and IL-2 is characteristic of Th_1 cells (Mosmann & Sad, 1997) which may play an important role in the regression of genital warts and SIL (Coleman *et al.*, 1994; see 1.5.3). However, as IL-2 and -4 were exogenous constituents of media supplements, the alternative experimental test was to determine INF-γ concentrations in available culture supernatants of peptide stimulated PBMCs.

Flat-bottomed 96 well plates (Corning) were coated overnight at 4°C with either 25 µl of human INF-γ diluted in carbonate bicarbonate buffer (13 ml 0.2M anhydrous sodium bicarbonate; 37 ml 0.2 M sodium hydrogen carbonate, pH 9.8; Sigma) at 8 concentrations: 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1} 1, 10^{1} 10^{2} and 10^{3} µg/L, or with 50 µl of supernatant from proliferative cultures diluted 1/10 (v/v) in carbonate bicarbonate buffer. Plates were then washed three times with 400 µl of 0.01% Tween™ (Sigma) in PBS (PBST)

and wells blocked for 20 min at room temperature with 400 µl of 1% bovine serum albumin (BSA; w/v) in PBST. Plates were washed three times with 400 µl of PBST then 50 µl of a 1:200 (v/v) dilution of a rabbit anti human INF-γ IgG antibody (Genzyme Diagnostics) in PBS were added to each well and incubated for 2 h at room temperature. Plates were washed three times with 400 µl PBST and 50 µl of a 1:250 (v/v) dilution of secondary goat anti rabbit IgG antibody conjugated to horse radish peroxidase (HRP; Sigma) in PBST were added to each well and incubated at room temperature for 2 h. The plate was washed three times with 400 µl PBST and 100 µl of orthophenylene diamine (OPD) substrate (Sigma Fast™; Sigma) were added to each well and colour was allowed to develop for 30 min, after which A_{490} values were determined on a plate reader (Labsytems).

2.2.7 Statistical tests.

Paired and unpaired, students' t-test (pt; ut), Fishers' exact (Fet) and the Mantel-Haenszel $_x^2$ (M-H $_x^2$) tests were used to assist the interpretation of data.

2.3 RESULTS

2.3.1 Detection of Human papillomavirus-16 nucleic acid

Two of the 75 samples, both SIL, were negative for β-globin DNA and hence excluded from HPV-16 DNA analyses. HPV-16 DNA was detected in cervical brush samples in 55 of the remaining 73 samples, 40 of 53 (75.5.4%) patients with cervical disease and in 15 of 20 (75%) asymptomatic women (Fet $p>0.05$). Of 21 HPV-16 E5 DNA positive samples, 19 were adequate for mRNA analyses (one asymptomatic and one L-SIL sample did not produce amplicons after RT-PCR for keratin, Table 2.5).

HPV-16 EmRNA was not detected in samples from asymptomatic patients (0 of 19: 0%) but was detected in 14 of 52 (26.9%) patients with cervical disease (Fet p=0.073; Table 2.5). The prevalence of HPV-16 EmRNA increased in parallel with non-malignant disease, 0 of 19 (0%) asymptomatic, 3 of 15 (20%) patients with L-SIL, 10 of 31 (32%) with H-SIL and 1 of 7 (14%) cancer patients were positive (M-H χ^2 test $p<0.0007$; Table 2.5).

Table 2.5 Detection of human papillomavirus-16 nucleic acid

		Total	Normal Cytology	Cervical Disease	L-SIL	H-SIL	Cervical Cancer
		75	20	55	15	33	7
HPV status	DNA	55 of 73 (75%)	15 of 20 (75%)	40 of 53 (75%)	13 of 15 (87%)	23 of 31 (74%)	4 of 7 (57%)
	EmRNA	14 of 71 (20%)	0 of 19	14 of 52 (27%)	3 of 14 (21%)	10 of 31 (32%)	1 of 7 (14%)

The HPV status is expressed as percentages of all positive samples, percentage of E6 or E5 positive samples and those with active infections characterised by EmRNA. 'Normal cytology' refers to asymptomatic women.

2.3.2 Peripheral blood mononuclear cell proliferation in response to stimulation by human papillomavirus type-16 E5 synthetic peptides

Proliferative PBMC responses to pooled HPV-16 E5 peptides were found for 16 of the 75 (21%) samples (Table 2.6). Overall, there were no differences between the frequencies of proliferation for women with normal cytology (4 of 20: 20%) or those with cervical disease (12 of 55, 22%: Fet: $p>0.05$). PBMC proliferation was most common in women with L-SIL (6 of 15, 40%) and lower in H-SIL (5 of 33, 15%) or cancer (1 of 7, 14%): this trend was significant (M-H χ^2 test, $p=0.027$: Table 2.6). Proliferative PBMC responses were more common in HPV-16 DNA positive subjects (15 of 56, 27% compared to 1 of 17, 6%, HPV-16 negative subjects, Fet $p>0.05$; Table 2.6).

However, proliferative responses in women with normal cytology were statistically independent of HPV-16 DNA status (3 of 15, 20%, HPV-16 DNA positive women and 1 of 5, 20%, in the HPV-16 DNA negative group: Fet $p>0.05$). For those with cervical lesions proliferative responses were restricted to HPV-16 DNA positive women (12 of 41: 29%, *c.f.* negative [0 of 12]: Fet $p=0.048$).

The frequency of PBMC proliferation for HPV-16 DNA positive women with cervical lesions was highest in L-SIL (6 of 14, 43%), lower in H-SIL (5 of 23, 22%) and cancer (1 of 4, 25%, M-H χ^2 test, $p>0.05$).

Five of 16 (31%) patients who exhibited PBMC proliferation were EmRNA positive and nine of 55 (16%) non-responders were EmRNA positive (Fet: $p>0.05$). There were no significant associations between PBMC responsiveness and EmRNA status in any of the subject groups (Fet & M-H χ^2 tests, all $p>0.05$: Table 2.6). All PBMCs tested responded to stimulation with concanavalin A. Equivalent analyses of responses to Concanavalin A failed to produce any differences (all $p>0.05$, Figure 2.4).

Table 2.6 Proliferative PBMC responses amongst subjects with/or without cervical lesions and with differing HPV-16 nucleic acid status.

	HPV-16 Status				
	Total	**HPV-16 DNA-**	**HPV-16 DNA+**	**HPV-16 E5 EmRNA-**	**HPV-16 E5 EmRNA+**
Normal Cytology	4/20 (20%)	1/5 (20%)	3/15 (20%)	4/19 (21%)	0/0 (0%)
Cervical Disease	12/55 (22%)	0/12 (0%)	12/41 (29%)	12/38 (32%)	5/14 (35%)
Total	16/75 (21%)	1/17 (6%)	15/56 (27%)	16/57 (28%)	5/14 (35%)
L-SIL	6/15 (40%)	0/1 (0%)	6/14 (43%)	5/11 (45%)	1/3 (33%)
H-SIL	5/33 (15%)	0/8 (0%)	5/23 (22%)	5/21 (24%)	3/10 (30%)
Cervical Cancer	1/7 (14%)	0/3 (0%)	1/4 (25%)	0/6 (0%)	1/1 (100%)

Data recorded as the number of PBMC responding over the total tested and in parentheses the percentage positivity in each group. **L-SIL**: low grade squamous intra-epithelial lesion; **H-SIL**: high grade squamous intra-epithelial lesion; HPV-16: subject positive (+) or negative (-) for HPV-16 DNA PCR; EmRNA: no of samples with proliferative T-cell response amongst those positive or negative for HPV-16 EmRNA. For 32 samples there were sufficient cells to assess reproducibility in duplicate, which was invariably high (*e.g.* subject 750, $r^2 = 0.82$).

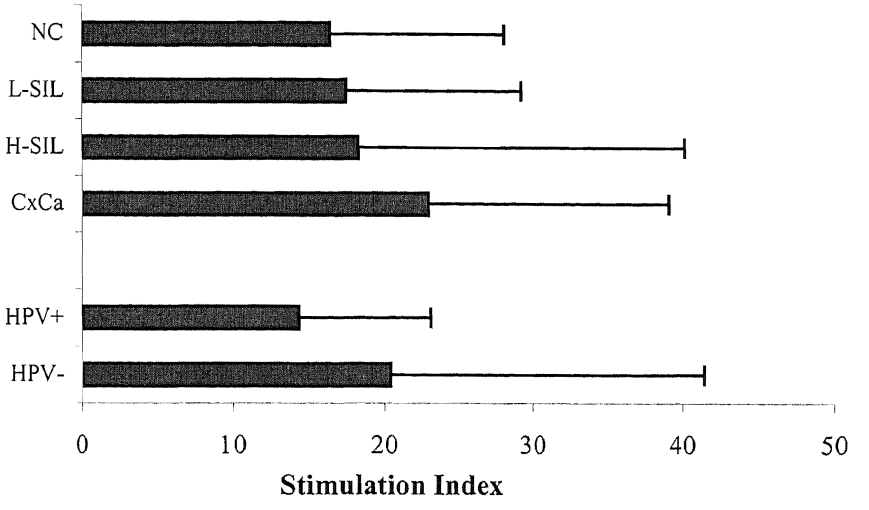


Figure 2.4 PBMC responses to concanavalin A. Patient PBMCs responding to concanavalin A were expressed as stimulation indices (SI). The SI between asymptomatic patients and those with varying degrees of cervical disease were not significantly different. The SI of those patients negative for HPV-16 DNA were not significantly different when compared with SI values from patients positive for HPV-16 DNA ($p>0.05$).

2.3.2.1 Detection of interferon gamma

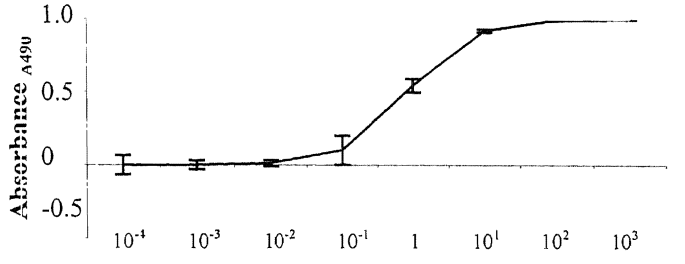
Cell-culture supernatants were available from 21 samples (8 from proliferative PBMC cultures). IFN-γ was detected in 4 of 8 (44%) samples from proliferative culture supernatants and in 3 of 11 (27%; Fet $p=0.34$) non-responsive subjects (Figure 2.5; Table 2.7). There were no associations between concordant detection of EmRNA and proliferative responses 4 of 22 (18.2%) patients were positive by RT-PCR: whereas EmRNA was only found in 9 of the 53 (17%) patients who did not exhibit proliferation (Fet: $p>0.05$).

Table 2.7 Characteristics of subjects with proliferative PBMC responses

Patient Number	Lesion	HPV-16 DNA	HPV-16 EmRNA	IFN-γ	SI>2.499	Highest SI
781	Cancer	+	+	Nt	2 of 26	8.9
681	H-SIL	+	+	+	2 of 24	21.4
724	H-SIL	+	+	Nt	3 of 80	2.9
628	H-SIL	+	+	-	7 of 24	12.5
790	H-SIL	+	-	Nt	1of 80	2.5
799	H-SIL	+	-	-	13 of 43	3.0
749	L-SIL	+	+	+	1 of 42	2.5
812	L-SIL	+	-	-	1 of 20	2.8
791	L-SIL	+	-	Nt	10 of 52	4.5
678	L-SIL	+	-	-	12 of 24	15.3
746	L-SIL	+	-	Nt	8 of 24	25.7
750	L-SIL	+	-	+	18 of 18	10.5
823	A	+	-	Nt	1 of 30	2.8
673	A	+	-	+	7 of 24	3.0
789	A	+	-	Nt	1 of 80	2.5
820	A	-	-	Nt	1 of 25	8.4

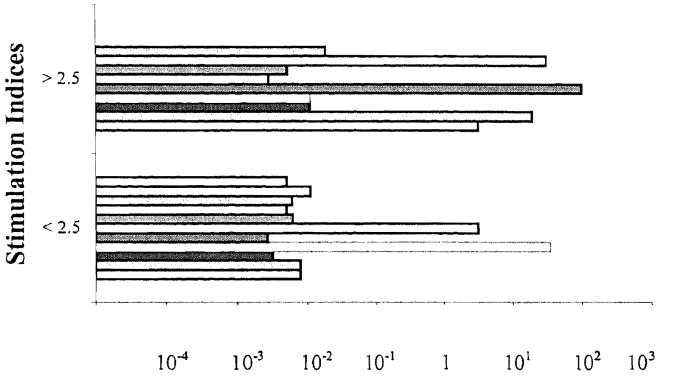
Patient Number: patient identification number; **A**: asymptomatic; **L-SIL**: low-grade squamous intraepithelial neoplasia; **H-SIL**: high-grade squamous intraepithelial neoplasia; HPV DNA: positive (+) or negative (-) by PCR for E6 and/or E5 DNA; EmRNA: results of an RT-PCR for EmRNA. IFN-γ enzyme-immunoassay: +: positive; - : not detected; **nt**: not tested; The number of cell lines with an SI greater than 2.499; **Highest SI**: highest SI value obtained in a single well.

a



Interferon-gamma mg/L

b



Interferon-gamma mg/L

Figure 2.5 Detection of interferon gamma in culture supernatants
a IFN-γ dose response curve. **b** IFN-γ in culture supernatants. Culture supernatants have been divided into those with stimulation indices greater than 2.5 and those with stimulation indices less than 2.5.

2.4 DISCUSSION

The initial hypothesis for this investigation suggested that women infected with HPV-16 were more likely to exhibit HPV-16 specific CMI which, if protective should be inversely correlated with disease severity. The data obtained in this chapter indicate that proliferative PBMC responses were inversely correlated with disease severity but not with the presence of HPV-16 DNA.

Testing for the presence of HPV-16 DNA by PCR revealed that 75% of the recruited women were positive, when primer pairs for both HPV-16 E6 and E5 ORFs were used. Due to the nature of the gynaecological clinics from which subjects were recruited, the majority of samples were expected to be positive for HPV-16 DNA. Similarly, previous studies performed on subjects from the same regional health authority had reported HPV-16 detection rates of 41-62% (Cavuslu *et al.*, 1996; Biswas *et al.*, 1997). However, both of these previous studies had included a larger number of asymptomatic women than the current study, and more importantly had failed to check for the presence of ORFs other than E5, thus explaining the lower HPV-16 DNA positivity. It is possible to miss HPV-16 infections using primers for E5 alone, considering it may not be expressed in H-SIL or cervical cancer where integration of the viral genome has occurred. The E6 and E7 ORFs however, are not present in all early region mRNA transcripts but are expressed abundantly in H-SIL and cervical cancer (Sherman *et al.*, 1992) and are therefore useful in detecting HPV infections in these lesions.

It was not possible to include a matched control group never exposed to HPV-16 due to reports of vertical transmission, HPV infection in virgins and to transient HPV infections where exposure to virus has occurred previously but where viral DNA is no longer detectable (see section 1.3.9).

It was anticipated that CMI may be more common in subjects with transcriptional active

HPV-16 infections. Samples were therefore tested for EmRNA, the detection of which is indicative of transcriptionally-active infections. There was a significant trend for EmRNA positivity with increasing grade of cervical lesion ($p<0.0007$). This correlates with the findings of Biswas *et al.*, (1997) who also reported that EmRNA prevalence increased with increasing severity of disease and was more frequently detected in samples from subjects with cervical disease than from samples from asymptomatic subjects.

The EmRNA assay is also an indirect measure of a high viral load compared to the HPV-16 DNA PCRs (E5 & E6) due to its comparative insensitivity, as the assay can only detect greater than 1,000 EmRNA copies (Biswas *et al.*, 1997). It is possible therefore that most subjects had low HPV-16 copy numbers, explaining the lack of association between EmRNA and proliferative PBMCs ($p>0.05$).

Patients PBMCs cultured with T-lymphocyte cytokines proliferated in response to HPV-16 E5 peptides. Proliferative PBMCs were detected primarily in samples from asymptomatic women. Similarly, proliferative T-cells have been detected in asymptomatic patients when stimulated with HPV E6 and L1 peptides and are thought to represent viral clearance (Strang *et al.*, 1990). This is in broad agreement with other studies which report between 18% to 47% of healthy subjects have T-lymphocyte responses to HPV-16 L1 and E7 proteins (Luxton *et al.*, 1996; Shepherd *et al.*, 1996). This data suggests that subjects with competent immune systems are able to mount effective CMI responses.

Although the proliferative PBMC responses in these asymptomatic subjects were independent of HPV-16 DNA status (both 20%: $p>0.05$), proliferative PBMCs in cervical disease were inversely correlated with severity of SIL ($p= 0.03$) and with the presence of HPV-16 DNA ($p>0.05$). This allows a comparison of data of Tsukui *et al.* (1996) who

reported an inverse correlation between *in vitro* CMI responses to HPV-16 E7 protein and severity of lesion. Similarly, proliferative T-lymphocyte responses to HPV-16 E7 were reduced in cervical cancer in comparison to SIL and healthy controls suggesting an altered functional balance between subsets of Th cells in HPV-16 infections (Luxton *et al.*, 1996). The same trend was seen for HPV-16 L1 protein where a decrease in Th_1 responses were seen in increasing grade of dysplastic lesion (Luxton *et al.*, 1997). Furthermore, it has been suggested that women with SIL are unable to respond to stimulation with HPV antigens as well as those women without cervical lesions (Nakagawa *et al.*, 1996). This implies that viral escape mechanisms may be used by HPVs to avoid recognition by the hosts' immune system.

A Th_1 response was also determined after stimulation of PBMCs with E6 and E7 proteins, where IL-2 production was greatest in cytologically normal women and declined with increasing severity of disease whilst responses to a recall influenza antigen were not significantly different between groups (Tsukui *et al.*, 1996).

An investigation of IFN-γ production in culture supernatants based on the same principle was performed in this study. IFN-γ was detected in supernatants from 4 of 8 patients with proliferative PBMC responses, suggesting a Th_1 response. Ideally, other Th_1-associated cytokines including TNF-α and cytokines associated with Th_2 type responses should also have been measured if larger quantities of culture supernatants had been collected. This may have helped to determine the type of Th response involved in HPV-16 E5 immunity. Other investigations have shown a slight decrease in the CD4+/CD8+ ratio in SIL and tumours especially when MHC-I antigens were lost suggesting a role for Th cells (Tay *et al.*, 1987; Hilders *et al.*, 1993). Protection in other chronic viral diseases such as HIV has also been shown to be mediated by Th_1 responses (Hilleman, 1994; Clerici *et al.*, 1994).

It would also have been worthwhile to measure mRNA for IL-6 in the cervical brush

samples, as the production of IL-6 has been shown to be related to severity of cervical disease (Tjiong *et al.*, 1999). The detection of IL-6 mRNA may have indicated which subjects were likely to exhibit proliferative PBMC responses and would have supported the link between IL-6 and disease severity.

It may be argued that these results reflect *in vitro* immunisation as PBMCs were cultured with E5 peptides, this is unlikely however, due to the inverse correlation between PBMC proliferation and disease status and the positive association between proliferation and HPV-16 DNA in lesions. It is currently thought that proliferative PBMCs could be the result of an effective immune response to HPV infections.

In conclusion, these studies suggest that patients with moderate to high-grade cervical lesions may have impaired proliferative PBMC responses to HPV-16 E5 peptides. In light of the HPV-16 E5 specific proliferative PBMC responses, the presence of antibodies to HPV-16 E5 should also be investigated. As individual HPV-16 E5 variants may have different immunogenic potential, further work should be directed at investigating common variants in this population.

Chapter Three

DNA Variation in the Human Papillomavirus Type-16 E5 Open Reading Frame and Detection of Reactive Serum Antibodies in Women with and without Cervical Disease

3.1 INTRODUCTION

The reference HPV-16 E5 ORF contains over 45 predicted restriction endonuclease cut sites. Comparison of these cut sites with established HPV-16 E5 variants revealed that three endonuclease cut sites within HPV-16 E5 would be disrupted in each variant DNA sequence (Seedorf *et al.*, 1985; Halbert & Galloway, 1988; Chan *et al.*, 1992). For example, DNA variation occurring at nucleotide position 3978 would result in the loss of a *Ssp*-1 cut site (3977-3982), variation at positions 3871 and 3873 would interrupt the *Xcm*-1 site (3871-3885) and variation at nucleotide 4076 would disrupt the *Nsp*-1 site (4076-4081). These endonuclease cut sites allow the identification of up-to 8 different HPV-16 E5 variants as well as the reference sequence (Table 3.1).

Similarly, the HPV-16 E6 ORF may also be subject to DNA variation, for example, an amino acid change at position 131 (characterised by the introduction of a second *Msp1* cut site) has been correlated with severity of lesion or the effectiveness of an immune response (Stern *et al.*, 1996). The introduction of an *Nsi* cut site at position 253 correlates with a second HPV-16 E6 variant which may also have indications for prognosis (Table 3.1).

The HPV-16 E5 and E6 amplicons from Chapter Two (see section 2.2.3) were investigated for DNA variation using restriction fragment length polymorphism (RFLP) analyses and DNA sequencing (Chan *et al.*, 1992). This was performed in order to determine the predominant local variants and to detect differences in serum reactivity to them, which may be correlated with disease severity.

As with investigations of CMI, most serological assays have focused on the HPV-16 E6 and E7 proteins and far less is known about antibody responses to other early HPV proteins. Antibody responses to E7 and E6 are thought to develop as a consequence of prolonged exposure to tumour because they are strongly associated with cancer with no

elevation of seropositivity in patients with SIL (Jochmus-Kudielka *et al.*, 1989; Mann *et al.*, 1990; Hamsikova *et al.*, 1994; Mandelson *et al.*, 1992; Muller *et al.*, 1992; Dillner *et al.*, 1993).

Table 3.1 Examples of restriction endonuclease cut sites on human papillomavirus type-16 E5 and E6 DNA

Restriction endonuclease cut sites	
HPV-16 E5 ORF	**HPV-16 E6 ORF**
SspI AAT↓ATT Position 3989 bp	*NsiI* ATGCA↓T Position 257 bp
XcmI CCANNNNN↓NNNNTGG Position 3878 bp	*MspI* C↓CGG Position 130 bp
NspI (**A/G**)CATG↓(T/C) Position 4080 bp	

bp=base pairs. For HPV-16 E5, PCR amplicons are of 273 base pairs in length, when that cut site remains intact two bands are produced after digestion with each particular restriction endonuclease. 1. ***SspI*** fragments are 152 and 121 bp, 2. ***XcmI*** fragments are 41 and 232 bp and 3. ***NspI*** fragments are 243 and 30 bp.

The need for a detailed investigation of HPV-16 E5 specific antibodies arises from the fact that only one previous study of this antigen has been reported. This previous study in humans reported weak IgG and IgA antibodies in HPV-16 positive patients with cervical neoplasia but, the same peptides were not tested on controls or asymtomatic women. The antigens used were 20-mer synthetic peptides corresponding to HPV-16 E5 and these might have been too long and folded tightly on themselves due to the hydrophobic nature of E5 (Figure 3.1).

For this study shorter 9-mer peptides were used to investigate serum reactivity to HPV-16 E5. Although still hydrophobic in nature, these peptides are more likely to be dispersed in solution than the peptides used by Dillner, (1992). To investigate serum reactivity to variant regions of HPV-16 E5, peptides of 13 amino acids were used, this length allowed the central (changed according to DNA variance) amino acid to be flanked by 6 non-variant amino acids (Figure 3.2). Subject's serum collected in section 2.2.2 was used for this investigation.

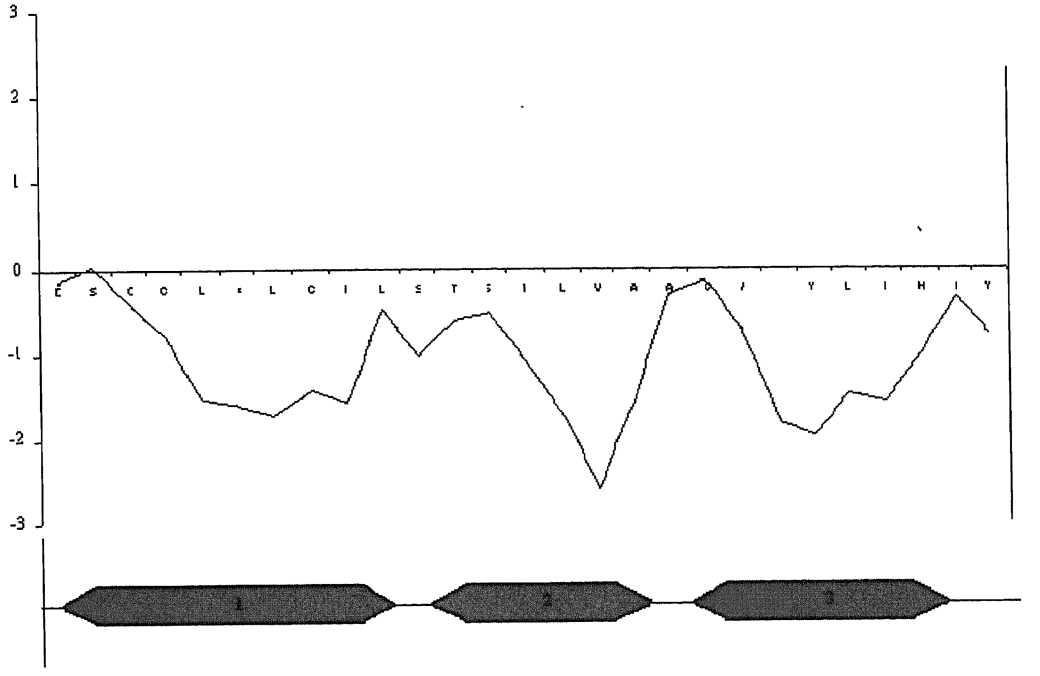


Figure 3.1 Hopp and Woods hydrophobicity plot of HPV-16 E5 Each point represents the mean hydrophobicity of five amino acids overlapped by one amino acid. The hydrophobicity values of all peptides used in this thesis are detailed in Appendix 3.

Control peptides

HPV-16 E2:13

NKVWEVHAGGQVILCPTSUF

HPV-16 E7:1

HGDTPTLHEYMLDLQPETTD

13-mer HPV-16 peptides with and without amino acid changes

Reference	Variants	Resulting endonuclease cut site
R1 ^{3}NLDTAS**T**TCCACF^{15}	**V1** ^{3}NLDTAS**P**TCCACF^{15}	***XcmI***
R2 ^{38}TYTSLI**L**LVLLLW^{50}	**V2** ^{38}TYTSLI**I**LVLLLW^{50}	***SspI***
R3 ^{59}CFIVYI**V**FVYIPL^{71}	**V3** ^{59}CFIVYI**I**FVYIPL^{71}	***NspI***

Figure 3.2 Synthetic human papillomavirus type-16 E2, E7 and E5 peptides Whole length HPV-16 E5 and the 9-mer peptides used in this study are described in Figure 2.2. The amino acid change in the variant 13-mer E5 peptides is compared to the reference peptides and the amino acid position is underlined and highlighted in bold. Variant peptides are referred to as V1-3 and peptides identical to the reference sequence of HPV-16 E5 are referred to as R1-3. The E2 and E7 peptides were included to allow comparisons between population and EIA variation. Superscripted numbers refer to amino acid position.

3.2 METHODS

3.2.1 Determination of human papillomavirus type-16 DNA variation in the E5 and E6 open reading frames

HPV 16 E6 and E5 PCR amplicons isolated from fourteen patients (see section 2.2.3) were analysed for DNA variance using RFLP. Individual variant sequences were confirmed by DNA sequencing.

3.2.1.1 Restriction fragment length polymorphism analysis of human papillomavirus type-16 E5 and E6 open reading frames

HPV-16 E5 positive PCR products (Table 2.5) were subjected to overnight digestion at 37°C with the *SspI*, *XcmI* or *NspI* endonucleases (Table 3.1). Briefly, 0.5 ml microcentifuge tubes were labelled *SspI*, *XcmI* or *NspI* and placed on ice. Three µl of non-purified PCR product were placed in each tube to which 2 µl of 10 × enzyme buffer were added, for tubes labelled *XcmI* and *NspI*, 2µl of BSA were also added. Molecular biology grade distilled water (H_2O) was added to each tube to obtain a final volume of 19 µl. Finally 1 µl of each enzyme was added to appropriately labelled tubes mixed gently and placed in a waterbath set at 37°C and incubated overnight (Table 3.2).

After incubation, 10 µl of 'digested' PCR products were mixed with 2 µl loading buffer and visualised after separation through a 2% agarose gel (see section 2.2.3; Figure 3.2). Similarly HPV-16 E6 amplicons were also subjected to overnight endonuclease digestion (Table 3.2).

Table 3.2 HPV-16 E5 and E6 enzyme digests

	HPV-16 E5			HPV-16 E6	
Enzyme	*Ssp-1*	*Xcm-1*	*Nsp-1*	*Msp-1*	*NSI*
DNA	3	3	3	5	5
Buffer	2	2	2	2	2
BSA*	2	———	———	2	———
Enzyme	1	1	1	1	1
dH_2O	12	14	14	10	12
Total volume (μl)	20	20	20	20	20

Units are expressed as μl and volumes of reactions were adjusted to enable overnight digestion of DNA. Enzymes were obtained from New England Biolabs and were supplied with the appropriate buffers. One μl of stock contained 10 units of enzyme.

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a **b**

Figure 3.3 Agarose gel showing restriction fragment length polymorphism patterns of HPV-16 E5 and E6 after digestion. **a**. arrow indicates 273 bp band. E5 lanes 1 & 5=uncut 2 & 6=*SspI* cut, 3 & 7=*XcmI* cut, 4 & 8=*NspI* cut. **b**.arrow indicates 482 bp band. E6 lane 1=uncut, 2=*MspI* cut, 3= *NsI* cut.

3.2.1.2 Ligation of human papillomavirus type-16 E5 and E6 PCR products to the pGEM®-T vector in preparation of DNA sequencing

HPV-16 E5 and E6 amplicons (from section 2.2.3) were ligated to the pGEM®-T plasmid vector (see Appendix 7) using the pGEM®-T TA cloning kit (Promega). The pGEM®-T plasmid was selected because it has overhanging thymidine at the 3' end which combines efficiently with the overhanging adenine at the 5' end of PCR amplicons (Mezei & Storts, 1994; Robles & Doers, 1994). The pGEM®-T plasmid contains T7 RNA and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase allowing amplification of inserted DNA after cloning. Insertion of the α-peptide allows recombinant clones to be directly identified by colour allowing blue/white screening in the presence of the substrate for β-galactosidase. Reactions were prepared in sterile 0.5 ml microcentrifuge tubes in which 1 µl of 10 × T4 DNA Ligase Buffer consisting of 300 mM Tris hydrochloride (pH 7.5), 10 mM magnesium chloride, 100 mM dithiothreitol and 10 mM adenosine triphosphate was added to 1 µl of pGEM®-T Vector (0.05 g/L), 2 µl of PCR product, 1 µl T4 DNA Ligase (3 Weiss units/ µl) and 5 µl H_2O. (All reagents were supplied with the kit). Control tubes contained 2 µl of pGEM®-T Vector Control instead of the relevant HPV-16 PCR product.

Reactions were incubated for 3 h at 15°C and then heated to 70°C for 10 min. The HPV-16 E5/vector ligation reaction was cooled to room temperature and transformed into JM109 (*Escherichia coli*, *E.coli*) cells (Promega). The JM109 cells were chosen as they were already prepared for transformation using the procedure of Hanahan (1983) and contained the $lacI^qZ\Delta M15$ mutation providing α-complementation of the β-galactosidase gene. This allows blue/white colour selection of recombinant colonies supplemented

with 5-bromo-4-chloro 3-indoyl-β-D-galactopyranoside (X-Gal; Alexis Corp) and isopropyl β-D-thiogalactopyranoside (IPTG; Alexis Corporation). The $lacI^q$ repressor produces 10 fold more lac repressor than found in most host strains ensuring repression of toxic fusion proteins (Lacks & Greenberg, 1977; Yanisch-Peron *et al.*, 1985).

JM109 cells (1×10^8 colony forming units per μg DNA) were gently thawed by placing on ice for 5 min, then a 50 μl aliquot was transferred to sterile 1.5 ml microcentrifuge tubes containing 2 μl of ligation products (from above) on ice. Cells and ligation mixture were gently mixed, left on ice for 20 min and heat shocked by placing on a heating block at 42°C for 40 sec and back on ice for 2 min. After 2 min, 1 ml of SOC media consisting of 0.2% (w/v) tryptone; 0.05% (w/v) yeast extract; 0.01 M sodium chloride; 0.0025 M potassium chloride; 0.02% (v/v) magnesium chloride; 0.02 M glucose; (Gibco) was added to each reaction tube and incubated at 37°C for 1 h.

Agar plates were prepared containing 25 ml Lauria Bertoni (LB) agar (1% (w/v) tryptone; 0.5% (w/v) yeast extract; 0.5% (w/v) sodium chloride; 1.5% (w/v) agar: Gibco) 25 μl of 10% ampicillin (Sigma), 20 μl of 12.5 mM X-Gal and 12.5 μl of 10mM IPTG. Plates were inoculated with 50 μl JM109 cells and incubated overnight at 37°C. Cells from single white recombinant colonies containing insert were checked for the correct insert by PCR using SP6 and T7 primers. PCR products of 421 bp were examined on a 1% (w/v) agarose gel after electrophoresis. Positive colonies were transferred into 3 ml LB medium consisting of 1% (w/v) tryptone; 0.5% (w/v) yeast extract; 0.5% (w/v) sodium chloride; (Gibco) and 0.1% (w/v) ampicillin, then incubated in a shaking mixer (Denley) at 37°C for 5 h.

DNA was extracted from the JM109 cells and purified using the QIAprep kit (Qiagen). This is based on the modified alkaline lysis method (Birnboim & Doly, 1979) and the adsorption of DNA onto silica in the presence of a high salt concentration (Chen &

Thomas, 1980; Marko *et al.*, 1982; Boom *et al.*, 1990). JM109 cells were centrifuged at 13,000 g for 5 min, the cell pellet was re-suspended in 250 µl of Buffer P1 consisting of 50 mM Tris-hydrochloride, pH 7.5, 10 mM EDTA and 100 µg/ml RNase in H_2O in a 1.5 ml microcentrifuge tube and inverted until no cell clumps were visible. To this, 250 µl of buffer P2 (cell lysis buffer) consisting of; 0.2 M sodium hydroxide and 1% (w/v) sodium dodecyl sulphate were added, and the tube was gently inverted to mix. To this 350 µl of buffer N3 consisting of 1.32 M aqueous potassium acetate, pH 4.8 were added and reactants were gently mixed by inversion followed by centrifugation at 13,000 g for 10 min.

Supernatants were applied to and centrifuged through QIAprep columns placed in 2 ml collection tubes (Qiagen). Eluates were discarded and columns were washed in 0.5 ml of buffer PB consisting of 4.5 M guanidine-hydrochloride in 40% (v/v) aqueous isopropanol. The columns were then placed in collection tubes and centrifuged at 13,000 g for 1 min. This was followed by a wash in 0.75 ml buffer PE consisting of 112 mM potassium acetate; 11.8 mM Tris-hydrochloride (pH 7.5), 0.6 mM EDTA and 55% (v/v) aqueous ethanol and centrifugation at 13,000 g for 1 min. The QIAprep columns were placed in 1.5 ml microcentrifuge tubes and 100 µl of H_2O was dispensed onto the QIAprep membrane to elute bound DNA. Columns were allowed to stand for 1 min at room temperature and then centrifuged at 13,000 g for 1 min. The DNA pellet was collected and rinsed in 70% (v/v) aqueous ethanol. The final solution containing purified DNA was centrifuged at 13,000 g for 30 min and the DNA pellet re-suspended in 10 µl of H_2O. The A_{260} was determined using a spectrophotometer (LKB) and DNA concentration was calculated using the following formula: 1 A_{260} Unit=50 µg DNA/ml (Freifelder, 1982). Samples were stored at -20°C until required.

3.2.1.3 Human papillomavirus type-16 E5 and E6 DNA Sequencing.

Extracted DNA (above) was sequenced using the AutoRead™ Sequencing Kit (Pharmacia). DNA concentration was adjusted to 1 g/L in H_2O and 32 µl aliquots were added to sterile microcentrifuge tubes containing 8 µl of 2 M aqueous sodium hydroxide. Tubes were vortexed gently and centrifuged briefly to collect solutions at the bottom of the tube then incubated at room temperature for 10 min after which 7 µl aliquots of 3 M aqueous sodium acetate (pH 4.8), 4 µl H_2O and 120 µl absolute ethanol were added. Tubes were gently vortexed and placed on dry ice for 15 min. Precipitated DNA was collected by centrifugation at 13,000 g for 15 min, carefully dried under vacuum for 10 min and then re-suspended in 10 µl of H_2O. To this 2 µl fluorescent primer (5 pmol) and 2 µl annealing buffer (1 M Tris-hydrochloride, pH 7.6; 100 mM magnesium chloride) were added. Tubes were gently vortexed and centrifuged 13,000 g to collect contents.

The annealing reaction was pre-heated to 65°C for 5 min and placed immediately at 37°C and incubated for 10 min. Tubes were allowed to cool to room temperature for 10 min after which 1 µl extension buffer consisting of 304 mM citric acid, 324 mM dithiothreitol and 40 mM manganese chloride was added as well as 3 µl of dimethyl sulphoxide.

To the cooled tubes 2 µl of diluted T7 DNA polymerase were added and mixed thoroughly, 4.5 µl of the purified HPV-16 E5 or E6 DNA were placed into each of the sequencing mixes and incubated at 37°C for 5 min. Sequencing mixes were prepared while the annealing reactions were cooling, briefly, 2.5 µl of A mix (5 µM ddATP; 1 mM dCTP; 1 mM dTTP; 1 mM dGTP, 50 mM sodium chloride; 40 mM Tris-hydrochloride, pH 7.6) were dispensed to a fresh microcentrifuge tube labelled A and placed on ice immediately. This was repeated for tubes labelled C, G and T where C

mix contained ddCTP; G mix contained ddGTP and T mix contained ddTTP instead of ddATP. After incubation 5 µl of stop solution (0.5% Dextran Blue 2000 in deionised formamide) was added to each tube and mixed gently. The reactions were heated to 90°C for 3 min and then quenched on ice. To each of four wells of a sequencing gel, 6 µl were loaded and electrophoresed. Data was analysed using a semi-automated sequencer (ALF; Perkin Elmer; Figure 3.3). Results obtained from the DNA sequencing were used to construct peptides for use in enzyme immunoassays.

3.2.2 Enzyme immunoassay to detect varicella zoster antibodies

A varicella zoster virus enzyme immunoassay (EIA) was include as a positive control for HPV-16 E5 EIA procedures. Due to the epidemiology of chicken pox, it was expected that most sera would have antibodies to this antigen. Flat bottomed 96 well plates (Corning) were coated overnight at room temperature with 50 µl/well of varicella zoster antigen (Boehringer Mannheim U.K.). Varicella zoster antigen was reconstituted and diluted in carbonate bicarbonate buffer consisting of 13 ml 0.2 M anhydrous sodium bicarbonate; 37 ml 0.2 M sodium hydrogen carbonate, pH 9.5 (Sigma) to give final dilutions of; 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024 and 1:2048. Plates were washed three times with PBS containing 0.01% Tween20 (sigma, PBST) in an automatic plate washer (Dynex Ltd.) and non-specific sites were blocked for 30 min at room temperature with 400 µl of PBS containing 1% (w/v) BSA. Plates were washed as above and 50 µl/well of 20 randomly selected patient's serum diluted 1:100 in PBST containing 0.1% (w/v) BSA were incubated in triplicate, overnight at room temperature in a humidified chamber. Plates were washed three times with PBST, after which 25 µl/well of HRP-labelled goat anti-human immunoglobulin (Sigma, specific for heavy chains) diluted to 1/250 in PBS were incubated in wells for 45 min at room temperature. Plates were

washed three times and 50 µl/well of Sigmafast™ OPD substrate were added for 10 min at room temperature in the dark. Reactions were terminated by the addition of 25 µl/well of 1 M sulphuric acid (Sigma) and A_{490} values were determined on a plate reader (Labsystems). All EIAs were performed in triplicate at various dilutions of sera to obtain optimal conditions.

3.2.3 Synthetic peptides

The antigens used in EIAs were synthetic peptides constructed by SPPS (see section 2.2.5). Serological assays employed full length HPV-16 E5 and a series of 9-mers which when overlapped by 4 amino acids correspond to the sequence of the reference isolate (Figure 2.3). Two additional positive control peptides were also constructed, these were E2:17 and E7:1 (Figure 3.2; Dillner, 1992). Following the identification of HPV-16 E5 variants in the study population, three corresponding pairs of 13-mer peptides were also constructed. These 13-mers correspond to the central amino acid substitution in the variants flanked by 6 amino acids on each side and the control peptides contained the reference sequence (Figure 3.2).

3.2.4 Coupling synthetic HPV-16 E5 to ovalbumin using carbodimide

To control for the differential binding of peptides to EIA plates, peptides were conjugated to ovalbumin. In a volume of 2 ml, synthetic HPV-16 E5 peptide was diluted 0.01 g/L in distilled water to which 1-ethyl-3(3-dimethylaminopropyl) carbodimide hydrochloride (Sigma) was added to give a final concentration of 0.1 g/L. The reaction was adjusted to pH 5.0 and incubated for 5 min at room temperature. A 2 ml volume of 1 g/L ovalbumin (Sigma) in water was added. This was incubated for a further 4 h at room temperature after which the reaction was stopped by the addition of sodium acetate

pH 4.2 (Sigma) at a final concentration of 100 mM (Doolittle 1986).

3.2.5 Enzyme immunoassays to detect reactive antibodies to full length human papillomavirus type-16 E5 peptides

Flat-bottomed 96-well plates were coated overnight at 4°C with 25 µl/well of a solution containing either (i) 1 g/L synthetic HPV-16 E5 conjugated to 10 g/L of ovalbumin, (ii) 11 g/L ovalbumin conjugated to itself or, (iii) 11 g/L ovalbumin in carbonate-bicarbonate buffer. Plates were washed and blocked as above (section 3.2.2). After the final wash 50 µl/well of patient's serum diluted 1:2, 1:10, 1:100 and 1:1000 in PBST containing 0.1% (w/v) BSA were incubated in triplicate for each antigen, overnight at room temperature in a humidified chamber.

The ovalbumin-ovalbumin conjugate was selected as a negative control for this EIA as any novel epitopes created by conjugation of ovalbumin were controlled for. Results were expressed as delta absorbance values (δA_{490}) for each sample (*i.e.* the mean absorbance A_{490} for the ovalbumin-ovalbumin conjugate subtracted from the mean A_{490} for the peptide-ovalbumin conjugate.

3.2.6 Enzyme immunoassays to detect reactive antibodies to human papillomavirus type-16 E5 9-mer peptides

Using the procedure described above (3.2.2) EIA plates were coated overnight with 1 g/L of individual synthetic 9-mers conjugated to 10 g/L ovalbumin. All patients sera were diluted 1:100 in PBST containing 0.1% (w/v) BSA were incubated in triplicate, overnight at room temperature in a humidified chamber. Antibodies specific for HPV-16 E5 9-mers were then detected as described above (3.2.2).

3.2.7 Statistical tests.

Paired and unpaired, students' t-test (pt; ut), Fishers' exact (Fet) and the Mantel-Haenszel $_x^2$ (M-H $_x^2$) tests were used to assist the interpretation of data.

3.3 RESULTS

3.3.1 Variance of the HPV-16 E5 and E6 open reading frames

Among the 14 patients tested for HPV-16 E5 DNA variance by RFLP, 2 (14%) were identical to the reference isolate, 8 of 14 (57%) were *Ssp-1* variants, 1 of 14 (7%) lost both *Xcm-1* and *Nsp-1* cut sites and one of 14 (7%) lost all three (*Xcm-1*, *Nsp-1* & *Ssp1*) cut sites (Table 3.3). Of the 16 patients tested for variance by RFLP in the HPV-16 E6 ORF all were identical to the reference isolate.

Table 3.3 Detection HPV-16 E5 and E6 variants

	Disrupted Cut Site	Number of Positives
HPV-16 E5	None	4 of 14 (28%)
	Ssp1	8 of 14 (56%)
	Xcm1+Nsp1	1 of 14 (7%)
	Xcm1+Nsp1+Ssp1	1 of 14 (7%)
HPV-16 E6	None	16 of 16 (100%)
	Msp-1	0 of 16 (0%)
	Nsi	0 of 16 (0%)

The above table shows the distribution of variance in the E5 & E6 ORFs in this study population. Ten samples were randomly selected and HPV-16 E5 DNA variance was confirmed using DNA sequencing (see section 3.3.2).

3.3.2 DNA sequencing

When DNA sequencing traces (Figure 3.3) of individual samples were compared, the DNA sequence in one direction invariably matched the DNA sequence in the opposite direction. Sequencing traces confirmed the reference DNA sequence showing no nucleic acid substitutions and no deletions of stop codons within HPV-16 E5 reference isolates. They confirmed nucleic acid substitution in the variant isolates, for example, *Ssp1*-A to C at position 3989; *Xcm1/Nsp1/Ssp1* A to a C at position 3878, G for a T at position 4080 and A to C at position 3989; *Xcm1/Nsp1* A to a C at position 3878 and G for a T at position 4080. When translated, these changes resulted in amino acid changes at amino acid position: (*Ssp1*) 44 L for a I; (*Xcm1/Nsp1/Ssp1*) 9 T for a P, 65 V for a I 44 L for a I and (*Xcm1/Nsp1*) 9 T for a P, 65 V for a I.

3.3.3 Serum responses to varicella zoster antigen

All 20 sera tested reacted strongly with *varicella* zoster antigen in all dilutions tested. The highest absorbance values were obtained at an antigen concentration of 1:32 (Figure 3.4).

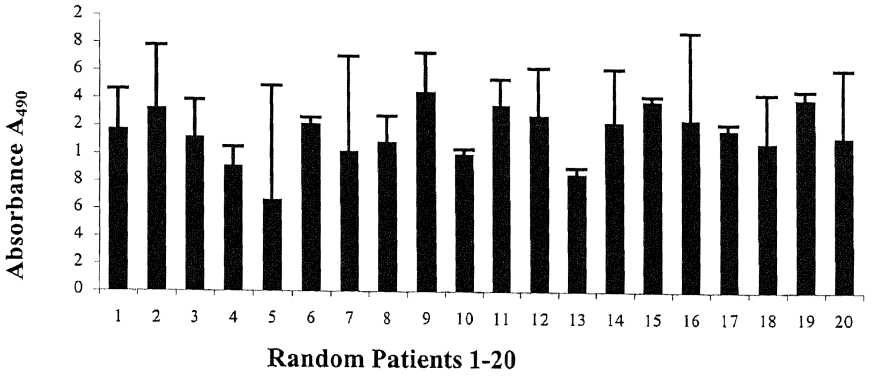

Figure 3.4 Serum reactivity to varicella zoster antigen Twenty randomly selected patients sera were tested to provide a positive control for EIA procedures. The assay was performed in parallel to the HPV-16 E5 whole length, E2 and E7 EIAs. Results are expressed as delta absorbance values

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3.3.4 Serum responses to full length HPV-16 E5, E2 and E7 peptides

None of 75 serum samples recognised full length E5 peptide in EIAs, but 8 of 75 (10.7%) sera bound to the control E2 peptide and 13 of 75 (17%) bound to the E7 peptide. Serum from six patients reacted with both E2 and E7 peptides, 4 of these had H-SIL, one had normal cytology and the other had cervical cancer. The magnitude of serum responses were not significantly different for either of the control peptides at a serum dilution of 1:100 ($p=0.6$: pt, Table 3.4).

Table 3.4 Serum reactivity to HPV-16 E2, E7 and whole length E5 peptides

Peptide	HPV-16 E5 (83 amino acids)	HPV-16 E2	HPV-16 E7
Total	0/75	8/75 (11%)	13/75 (17%)
Normal Cytology	0/20	2/20 (10%)	1/20 (5%)
Cervical Disease	0/55	6/55 (11%)	12/55 (22%)
L-SIL	0/15	1/15 (7%)	4/15 (27%)
H-SIL	0/33	4/33 (12%)	7/33 (21%)
Cervical Cancer	0/7	1/7 (14%)	1/7 (14%)
HPV-16 +	0/55	4/55 (7%)	10/55 (18%)
HPV-16 -	0/18	4/18 (22%)	3/18 (17%)

3.3.5 Serum reactivity to HPV-16 E5 9-mer peptides

Sixteen of 75 (21%) sera reacted with one or more 9-mer peptides corresponding to the least hydrophobic regions of HPV-16 E5 (amino acids 1-8; 5-13; 30-38; 45-53 and 55-63). Prevalence of seropositivity to one or more peptides was greater in samples from patients with cervical disease than those who had normal cytology and this difference was significant when peptide 45-53 was used (Fet; p=0.01; Table 3.5). When data were

analysed with respect to severity of disease, seropositivity rates were generally lower in the normal cytology group and usually increased in parallel with severity of disease so that when H-SIL (but not L-SIL) were compared to normal cytology women differences were significant for 3 peptides (1-8 H-SIL 10 of 33 [30%], NC 1 of 20 [5%]: p=0.04; 45-53 H-SIL 11 of 33 [33%]; NC 0 of 20 [0%]: p=0.004; 55-63 H-SIL 9 of 33 [27%], NC 0 of 20 [0%]: p=0.01). When seropositivity to peptides was compared between HPV-16 DNA positive and negative subjects, the differences were not statistically significant (p>0.05).

3.3.6 Reactivity to Human Papillomavirus Type 16 E5 variant peptides

To determine differences in serum cross reactivity between variant and reference regions of HPV-16 E5 all sera were re-tested 13-mer peptides corresponding to each of the variant regions of HPV-16 E5 peptide V1, R1, V2, R2, V3 and R3 (Table 3.6). Only 8 sera reacted to any peptide, V1 1 sera (L-SIL), V3 2 sera (H-SIL), R1 2 sera (NC+H-SIL) and R3 3 sera (NC+H-SIL). Reactivity to all three peptides containing the amino acid substitution were significantly greater (p=0.05;ut) than reactivity to 'reference peptides' using a total immunoglobulin antibody.

Table 3.5 Serum antibodies to HPV–16 E5 9-mer peptides

Peptide	^{1}MTNLDTAS^{8}	^{5}DTASTTCCA^{13}	^{30}RPLLLSVST^{38}	^{45}LVLLLWITA^{53}	^{55}SAFRCFIVY^{63}
Total	16/75 (21%)	12/75 (16%)	9/75 (12%)	13/75 (17%)	10/75 (13%)
Normal Cytology	1/20 (5%)	3/20 (15%)	0/20 (0%)	0/20 (0%)	0/20 (0%)
Cervical Disease	15/55 (27%)	9/55 (16%)	9/55 (16%)	13/55 (24%)	10/55 (18%)
L-SIL	3/15 (20%)	1/15 (7%)	2/15 (13%)	1/15 (7%)	1/15 (7%)
H-SIL	10/33 (30%)	7/33 (21%)	6/33 (18%)	11/33 (33%)	9/33 (27%)
Cervical Cancer	2/7 (29%)	1/7 (14%)	1/7 (14%)	1/7 (14%)	0/7 (0%)
HPV-16+	8/55 (14%)	7/55 (13%)	5/55 (9%)	8/55 (15%)	7/55 (13%)
HPV-16-	8/18 (44%)	5/18 (26%)	4/18 (22%)	5/18 (28%)	3/18 (17%)

EIA assays using peptides 1 to 8, 5 to 13, 30 to 38, 45 to 53 and 55 to 63 were highly reproducible for 10 sera (5 EIA positive and 5 negative) were tested three times in triplicates against these peptides (median r^2= 0.86). Responses to these peptides were predominant in women with cervical disease at a serum dilution of 1:100. Superscripted numbers refer to amino acid position of peptide sequences compared to whole length HPV-16 E5.

Table 3.6 Serum reactivity to variant HPV-16 E5 13-mer peptides

Peptide	V1	P1	V2	P2	V3	P3
Total	37/75 (49%)	6/75 (8%)	13/75 (17%)	5/75 (7%)	5/75 (7%)	15/75 (20%)
Normal Cytology	9/20 (45%)	0/20 (0%)	1/20 (5%)	4/20 (20%)	1/30 (3%)	4/20 (20%)
Cervical disease	28/55 (51%)	6/55 (11%)	12/55 (22%)	1/20 (5%)	4/55 (7%)	11/20 (55%)
L-SIL	10/15 (67%)	1/15 (7%)	4/15 (27%)	0/15 (0%)	0/15 (0%)	1/15 (7%)
H-SIL	15/33 (46%)	4/33 (12%)	7/33 (21%)	0/33 (0%)	3/33 (9%)	8/33 (24%)
Cervical cancer	3/7 (43%)	1/7 (14%)	1/7 (14%)	1/7 (14%)	1/7 (14%)	2/7 (29%)
HPV-16 +	31/55 (56%)	5/55 (9%)	8/55 (15%)	1/55 (2%)	3/55 (5%)	7/55 (13%)
HPV-16 -	6/18 (33%)	1/18 (6%)	5/18 (28%)	4/18 (22%)	2/18 (11%)	8/18 (44%)

3.4 Discussion

HPV-16 E5 and E6 isolates were investigated for DNA sequence variation and correlated with disease status or the effectiveness of an immune response. Certain E6 variants have been associated with increased severity of lesions but these were not detected in this cohort (Stern *et al.*, 1996). It is likely that these E6 variants are not representative of this study population or may not have been detected due to the small number of women with H-SIL or cervical cancer. However, 3 E5 variants were detected and analysed for significant differences in proliferative PBMC responses, serology or disease status but none were found. Synthetic 13-mer peptides accommodating these amino acid variants were designed with the variant amino acid located in the centre of the peptides for use in EIA. Re-testing of the sera against these peptides did result in significant changes when total immunoglobulin was detected. Overall, the A_{490} values for all 13-mer peptides were much higher than A_{490} values to 9-mer peptides, which may be a reflection of the length of antigen used. Although the number of variants was limited in this investigation, other studies in the department have indicated that nucleotide variation of the E5 ORF is common and that coding changes occur as a result.

Whole length HPV-16 E5 was not recognised by any sera in EIAs. This effect may be due to the hydrophobicity of HPV-16 E5 which may compromise its antigenicity. There was serum reactivity to two control peptides, E2 and E7 which were included to allow comparisons to previously published data (Dillner, 1992). Although serum reactivity for the E2 peptide were eight times lower than in the previous study, reactivity to the E7 peptide was similar in this study responses. These differences may reflect variations in methodology used, as all peptides in this study were conjugated to ovalbumin and results were expressed as delta absorbance values.

To overcome the problem of hydrophobicity, five ovalbumin conjugated 9-mer peptides

corresponding to the least hydrophobic regions of E5 were selected for use in subsequent EIAs. EIA positivity to these 9-mer peptides were more common in patients who were negative for HPV-16 DNA.

It is possible therefore, that seropositivity for HPV-16 E5 may be a marker of an effective immune response that results in cell lysis causing release of E5 and the production of antibodies. The presence of HPV-16 E5 specific antibodies may be protective against the development of cervical cancer.

Serum reactivity to variant sequences did differ significantly from reference sequences suggesting that amino acid change does alter serum reactivity to HPV-16 E5. It could also be that patients may have been infected with more than one HPV-16 virus and have already seen both variant and reference sequences of E5. Alternatively, as cross presentation of antigen is dose dependant the low level of HPV antigen may not have been sufficient for cross presentation (Carbone *et al.*, 1998).

When serological data were compared with PBMC responses from the previous chapter, only two patients were positive in both assays. Only two proliferative PBMC patients were concordantly EIA positive (cases 746; L-SIL & 820; asymptomatic). When subjects were subdivided according to the correlation of proliferative PBMCs and antibody positivity (T+B+: putative Th_2 responses) or proliferative PBMCs and antibody negative (T+B-: Th_1 responses), a trend towards greater Th_1 responses in patients with more severe cervical disease was seen. This is in agreement with Coleman *et al.,* (1994) who suggested that Th_1 responses are important in the regression of cervical disease. These results generally support the observations of decreased Th_1-like responses to HPV-16 antigens with increasing grade of neoplasia (Tsukui *et al.*, 1996; Coleman *et al.*, 1994; Luxton *et al.*, 1996).

Infection with common skin and genital HPVs is generally chronic even in late childhood

when the immune system is at its peak (Frazer & Tindle, 1992). This chronic nature suggests that there is a problem with papillomavirus antigen presentation to the immune system. As with rhinoviruses to which the immune system produces very little immune response during natural infection because they are not presented at the right place or in the right way or with HIV and herpes which are immunogenic but have host evasion mechanisms. The next step towards the characterisation of immune responses would be to investigate the *in vivo* responses to immunisation with HPV-16 E5. This could provide data regarding antigenic processing and presentation by a competent immune system.

Chapter Four

Immune Responses to Human Papillomavirus Type-16 E5 in Mice

4.1 INTRODUCTION

The HPV-16 E5 specific immune responses detected in Chapters Two and Three appear to be correlated with disease severity. For example, proliferative PBMC responses were detected primarily in subjects with normal cytology and in Chapter Three, serum antibodies were detected in those subjects who were negative for HPV-16 DNA. It may be possible therefore, that HPV-16 E5 is effectively presented to cells of the immune system and is immunogenic in some subjects.

In order to investigate the immunogenic properties of HPV-16 E5, mice were immunised with synthetic peptides using three different immunisation procedures (Table 4.1), devised to investigate whether both cell mediated and humoral immune responses to HPV-16 E5 were inducible. The presence of HPV-16 E5 reactive antibodies were investigated as evidence of humoral immunity. HPV-16 E5 specific spleen cell proliferation and CTL responses were measured as evidence of CMI.

As CTL responses were detected after immunisation procedure three, peptide-binding assays were performed to map the HPV-16 E5 epitopes which bind to MHC-I molecules.

4.2 METHODS

Synthetic peptides were used as immunogens and were constructed using SPPS (see section 2.2.5). Full length HPV-16 E5, 9-mers (Figure 2.2) and 6-mers which when overlapped by two amino acids corresponded to full length HPV-16 E5 (Figure 4.1) were used. Peptides of this length were chosen as fragments of 5-15 amino acids are generated after *in vitro* digestion of whole proteins by proteosome units and may represent MHC-I epitopes (Wenzel *et al.*, 1994; Boes *et al.*, 1994; Dick *et al.*, 1994; Kuckelkorn *et al.*, 1995; Groettrup *et al.*, 1995). Studies have shown that 9-mers bind to MHC-I 100 to 1000 fold higher than shorter or longer peptides (Kubie, 1997). Indeed, a 9-mer peptide

was also found to have a ten fold higher affinity to pooled antisera from subjects who had recovered from Hepatitis B (Kuby, 1997).

The systematic testing of short overlapping synthetic peptides for MHC-I binding may identify peptides that are recognised by MHC restricted CTL responses. MHC-I binding epitopes contain amino acids whose residues fit into pockets of a peptide-binding cleft. The 6-mer peptides allow determination of anchor residues which are usually hydrophobic leucines or isoleucines (Kubie, 1997). Conserved anchor residues exist in peptides to various MHC-I molecules and permit the prediction of peptide binding to a particular MHC molecule and this may help in designing effective synthetic peptide vaccines.

Using an epitope mapping kit, SPPS reactions were used to prepare peptides onto high-density acrylic grafted polyethylene rods (Geysen *et al.*, 1987). These rods were designed in the format of a 96 well microtitre plate and were used in EIAs. Reactions to remove Fmoc groups present on the tips of the rods and subsequent reactions involving addition of amino acids were carried out in wells of a Teflon block. Peptides were not cleaved from the rods and were used *in situ* during EIAs. Peptides present on Pepscan™ pegs were 5-mers overlapping by 2 amino acids (Figure 4.2), their short length enables epitope mapping of reactive sera.

Table 4.1 Immunisation procedures used in this chapter

Procedure Number	Antigen	Number of Mice	Tests
Procedure 1	full length HPV-16 E5	9	Serum antibody & Spleen cell proliferation
	DPBS	4	
Procedure 2	full length HPV-16 E5	3	Serum antibody
	full length HPV-16 E5 mixed with ovalbumin	3	"
	full length HPV-16 E5 conjugated to ovalbumin	3	"
	DPBS	3	"
Procedure 3	full length HPV-16 E5	3	CTL & Mucosal antibody
Antigens entrapped within ISCOMs complexes	full length HPV-16 E5 conjugated to ovalbumin	3	"
	full length HPV-16 E5 mixed with ovalbumin	3	"
	9-mer peptides	3	"
	9-mer peptides conjugated to ovalbumin	3	"
	9-mer peptides mixed with ovalbumin	3	"
	Ovalbumin	3	"
	DPBS	3	"

Various immunological investigations of HPV proteins have used murine models and some have even led to clinical trials of vaccines. Such models have shown partial protection against BPV-1 E5, E6 and E7 and protection against HPV-16 E6 and E7 proteins using recombinant vaccinia virus (Meneguzzi *et al.*, 1991), transfected cells (McLean *et al.*, 1993; Chambers *et al.*, 1994) or synthetic peptides (Chen *et al.*, 1992; Feltkamp *et al.*, 1993; Tindle *et al.*, 1991). HPV-16 E5 specific CTL were investigated after immunisation procedure 3 to determine whether HPV-16 E5 peptides are processed by MHC-I molecules. All antigen preparations were emulsified in equal volumes of Freunds incomplete adjuvant. **DPBS**= Dulbeccos phosphate buffered saline, **ISCOMs**= immunostimulatory complexes, **CTL**= cytotoxic T-lymphocytes

6-mer peptides

^{10}TCCACF^{15} ^{22}LLCVCL^{27} ^{34}LSVSTY^{39} ^{46}VLLLWI^{51} ^{58}RCFIVY^{63} ^{70}PLFLIH^{75}

^{6}TASTTC^{11} ^{18}CFCVLL^{23} ^{30}RPLLLS^{35} ^{42}LIILVL^{47} ^{54}ASAFRC^{59} ^{66}FVYIPL^{71} ^{78}ATNQIY^{83}

^{1}MTNLDTA^{7} ^{14}CFLLCF^{19} ^{26}CLLIRP^{31} ^{38}TYTSLI^{43} ^{50}WITAAS^{55} ^{62}VYIIFV^{67} ^{74}IHTHAT^{79}

Pepscan™

^{1}MTNLD$_{1\text{-}5}$ ^{4}TCCAC$_{10\text{-}14}$ ^{7}FCVLL$_{19\text{-}23}$ ^{10}LIRPL$_{28\text{-}32}$ ^{13}STYTS$_{37\text{-}41}$ ^{16}VLLLW$_{46\text{-}50}$ ^{19}SAFRC$_{55\text{-}59}$ ^{22}IIFVY$_{64\text{-}68}$ ^{25}LIHTH$_{73\text{-}77}$

^{2}LDTAS$_{4\text{-}8}$ ^{5}ACFLL$_{13\text{-}17}$ ^{8}LLCVC$_{22\text{-}26}$ ^{11}PLLLS$_{31\text{-}35}$ ^{14}TSLII$_{40\text{-}44}$ ^{17}LWITA$_{49\text{-}53}$ ^{20}RCFIV$_{58\text{-}62}$ ^{23}VYIPL$_{67\text{-}71}$ ^{26}THATN$_{76\text{-}80}$

^{3}ASTTC$_{7\text{-}11}$ ^{6}LLCFC$_{16\text{-}20}$ ^{9}VCLLI$_{25\text{-}29}$ ^{12}LSVST$_{34\text{-}38}$ ^{15}IILVL$_{43\text{-}47}$ ^{18}TAASA$_{52\text{-}56}$ ^{21}IVYII$_{61\text{-}65}$ ^{24}PLFLI $_{70\text{-}74}$ ^{27}TNQIY$_{79\text{-}83}$

LAFLT –control sequence

Figure 4.1 Synthetic human papillomavirus type-16 E5 peptides Amino acid sequences of the peptides used in this study, 6-mers were constructed with a two amino acid overlap. Superscripted numbers refer to amino acid sequences. The Pepscan™ is made up of 5-mer peptides, superscripted numbers in bold refer to rod number, whilst subscripted numbers refer to amino acid sequences which make up each epitope. The LAFLT sequence was constructed as a negative control and was prepared on rod 28..

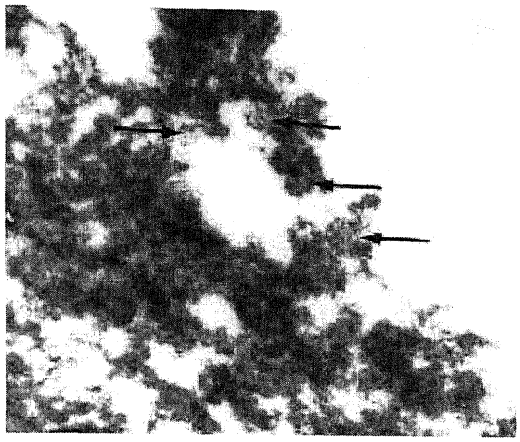
4.2.1 HPV-16 E5 containing immunostimulatory complexes (ISCOMs)

To prepare ISCOMs, 0.5 mg of the required HPV-16 E5 peptide was added a solution of 1 ml of carbonate buffer pH 9.0, containing 5% (w/v) sodium deoxycholate (Sigma) and 10 % (v/v) dimethyl sulphoxide. To this, 100 µl of chloroform (Sigma) containing 1 g/L of cholesterol (Sigma) and 1 g/L of phosphatidyl choline (Sigma) was added. The chloroform was removed under vacuum with constant agitation at 37°C, then 1 g/L of Quil A (Sigma) was added to the solution. This was dialysed for three days at room temperature against 50 mM Tris-hydrochloride buffer at pH 8.0 containing 0.001% (w/v) of the anti bacterial agent thimerosal (Sigma) to allow formation of the ISCOMs.

After formation of ISCOMS the preparation was centrifuged at 30,000 g through a layer of 10% (w/v) sucrose in water onto a 40% (w/v) sucrose in water cushion for 18 h at 20°C. The sucrose solutions were prepared in 50 mM Tris-hydrochloride buffer at pH 8. ISCOMS (seen as a red opaque band at the interface) were harvested and then dialysed against PBS pH 7 at 4°C overnight. The presence of ISCOMs was determined by transmission electron microscopy (Figure 4.2).

4.2.2 Preparation of injections

In a class II laminar flow hood, 10 µg of antigen (Table 4.1) were added to 1 ml of filter sterilised 10% (v/v) glacial acetic acid in Dulbeccos phosphate buffered saline (DPBS, Sigma) and emulsified with 1 ml of Freunds incomplete adjuvant (Sigma). Where ISCOMs were used as antigen mice were injected with ISCOM preparation emulsified with Freunds incomplete adjuvant.



0.1 μm

Figure 4.2 Electron micrograph of ISCOMs A transmission electron microscope HU12A (Hitachi) was used to prepare these images. 25 μl of the ISCOM preparation was allowed to dry on copper grids. The excess was removed and 25 μl of a negative uranium salt dye was used to stain the preparation. Arrows indicate the presence of formed ISCOMs. The above figure is magnified 125,000 times.

4.2.3 Immunisation of mice

Six-week-old female Balb/c mice (Harlan Olac Ltd., U.K.) were anaesthetised in an inhalation chamber (Jencons Scientific Ltd., U.K.) containing cottonwool saturated with Enflurane™ (Abbott laboratories, Ltd.). Mice were immunised subcutaneously with a total of 50 μl of the appropriate injection suspension at two sites near the nape of the neck. Control mice were kept in neighbouring cages and immunised with equal volumes of DPBS and Freunds incomplete adjuvant emulsion. Immunisations were repeated at weeks two and four. At six weeks mice were anaesthetised as above and exsanguinated from the heart using a 21 gauge needle attached to a 1 ml syringe (Sterilin) and killed by stunning

and cervical dislocation. In all three immunisation procedures DPBS was used as a negative control, in procedures 2 and 3, ovalbumin provided a positive control.

4.2.4 Collection of samples

Whole blood was permitted to clot and then centrifuged at 500 g for 5 min to collect serum. Peritoneal cells were harvested under sterile conditions for use as autologous feeder cells as follows. Using a sterile needle and syringe, 8 ml of RPMI-1640 supplemented with 10% FBS, 0.04 g/L gentamycin (Sigma) and 0.12 g/L benzylpenicillin (Sigma) was injected into the peritoneal cavity. The abdomen was then gently massaged, to bring cells into suspension, peritoneal fluid was collected by aspiration of the media. Cell suspensions were centrifuged at 150 g for 10 min and cell number was adjusted to 1 $\times 10^5$/ml in FBS containing 10% dimethyl sulphoxide (v/v). Cells were then frozen to -70°C at a rate of -1°C per min in 1.8 ml cryotubes (Corning) using a freezing chamber and stored in liquid nitrogen until required.

Once peritoneal cells had been harvested, spleens from each mouse were removed and placed into sterile universals half-filled with wash media consisting of, Hanks balanced salt solution; 1% (v/v), 1 M Hepes buffer, 0.04 g/L gentamycin and 0.12 g/L benzylpenicillin. Spleens were removed from wash media in a class II laminar flow hood (MDH) and teased through a sterile wetted wire mesh using the plunger of a 10 ml syringe. Resulting cell suspensions were washed through the mesh using 5 ml of culture media consisting of Dulbeccos modified Eagles media, (DMEM), 10% FBS, 2 mM l-glutamine, 100,000 U/L benzylpenicillin and 0.1 g/L streptomycin sulphate.

Cellular aggregates were allowed to settle and the single cell suspension was transferred to a 15 ml centrifuge tube (Corning) and centrifuged at 150 g for 10 min. Pelleted cells

were re-suspended in 10 ml culture media, centrifuged for a further 10 min at 150 g and then placed in 25 cm^3 tissue culture flasks (Corning).

When mice had been killed after immunisation procedure 3, additional samples were collected for investigations of HPV-16 E5 specific mucosal (IgA) responses. These included faecal pellets expelled after death. These were weighed and re-suspended in DPBS at a concentration of 5 g/L (w/v). Cotton tipped swabs (Johnson & Johnson, U.K.) were used to collect mucous from oral and vaginal cavities and were suspended in 1 ml DPBS.

4.2.5 EIA for detection of antibodies to human papillomavirus type-16 E5 peptides

Sera collected from mice were diluted in PBS to dilutions of: 1:50; 1:100, 1:200, 1:400, 1:800, 1:1600 and 1:3200. Aliquots of 100 µl were added to each well of a 96 well plate previously coated with whole length HPV-16 E5 (see section 3.2.8). After 1 h at room temperature plates were washed three times in PBST and 50 µl of HRP labelled polyvalent goat anti-mouse antibody (Sigma) were added at a dilution of 1:250. The plates were then incubated for 1 h at room temperature. The rest of the EIA procedure was previously described in section 3.2.2.

4.2.6 Pepscan™ immunoassays

To inactivate non specific binding sites, rods were immersed in 250 µl of 1% BSA (w/v) in PBST in 96 U-well polycarbonate microtitre plates (Corning) for 1 h at room temperature on a rocking platform (Denley A600). After washing in running tap water, rods were placed in wells of a fresh 96 U-well microtitre plate containing 50 µl of immunised or control mouse serum, diluted 1:100, and left to incubate overnight at 4°C in a humidified chamber.

Rods were washed again under running tap water and placed in a fresh 96 well plate containing 50 µl per well of goat anti-mouse antibody conjugated to HRP (Sigma) diluted to 1:250 in PBS (v/v) and incubated for 1 h at room temperature. Rods were washed thoroughly in tap water and placed in a fresh plate containing 100 µl/well of 2,2 azino-bis(3-ethylbenzthia-zoline)-6-sulphonic acid (Sigma). They were then left to incubate for 1 h at room temperature on a rocking platform, to allow colour development. Rods were removed and the A_{405} of each well were determined using an automatic plate reader (Labsystems). The A_{405} value obtained for the control peptide (LAFLT, Figure 4.2) was subtracted from those obtained for HPV-16 E5 peptides.

To re-use Pepscans™ bound antibody was removed by placing the rods in a hot (55°C) aqueous solution containing 10% (w/v) sodium dodecyl sulphate (Sigma) and 2% (v/v) 2-mercapto-ethanol (BDH). They were then sonicated for 30 min in a water bath (Grant, UK) and washed three times in hot water at 56°C and a final rinse in methanol (Sigma).

4.2.7 Generation of antigen specific splenic T-lymphocyte lines by continuous stimulation

Cells isolated from spleens were adjusted to 2×10^5/ml viable cells in 5 ml of RPMI 1640 containing 1 mM pooled 6-mer peptides, 10,000 U/L IL-2 and 20 g/L of concanavalin A. Cells were cultured using the method of Good *et al.*, 1987 and were placed in 25 cm^3 flasks in a humidified 37°C incubator with 5% CO_2 in air (v/v) for three days. On the fourth day cultured spleen cells were collected by centrifugation as above and washed twice in culture media. Cells were counted and cell number was adjusted to 1×10^5 viable cells/ml. Aliquots of 225 µl were placed in 96 round well tissue culture plates (Corning) with 10,000 U/L IL-2, 20 g/L concanavalin A and 1 mM pools of four 6-mer peptides. Cells were incubated at 37°C in a humidified incubator with 5% CO_2 (v/v) in air

for 7 days (Good *et al.*, 1987). In three fresh plates 100 µl volumes of 1×10^5 mitomycin C treated autologous feeder cells (see section 4.2.5) were pipetted into each well of three 96 round well microtitre plates into which 75 µl aliquots of stimulated cells were added. Two of these plates were stimulated with 25 µl of 1 mM pools of four 6-mer peptides and the third received 25 µl of culture medium only (negative control). Plates were incubated for 5 days in a humidified incubator at 37°C with 5% CO_2 in air (v/v), after which cells were collected by aspiration. Cells were then washed twice in culture media and re-suspended as before with stimulation media and incubated for a further 7 days. Cells were tested for HPV-16 E5 specific proliferation using the MTT assay and results were expressed as SI (see section 2.2.6.1).

4.2.8 Cytotoxic T-lymphocyte assay

The murine thyoma cell line p815 (ECACC) shares the same $H2^d$ haplotype as Balb/c mice and were used as target cells in CTL assays. After immunisation, Balb/c mice have been shown to generate CTLs capable of lysing p815 cells indicating that the method used in this chapter is an effective one (Zhou *et al.*, 1991).

p815 cells were pulsed with HPV-16 E5 9-mers to induce macropinocytosis (see Appendix 4). Briefly, 1×10^5/ml p815 cells were suspended in 2 ml RPMI 1640 medium, containing 0.5 M sucrose, 20 g/l polyethylene glycol 1000™ and 2 mM HPV-16 E5 9-mers. After incubation at 37°C for 10 min in a humidified atmosphere containing 5% CO_2 (v/v) in air, cells were washed once in a solution of 3 parts Hams' F12 media (Sigma) and 2 parts tissue culture grade water (Sigma). Cells were then counted and re-suspended at 2×10^5/ml in RPMI/FBS.

Aliquots of 50 µl were then dispensed into wells of a 96 U-well microtitre plate. To each well an equal volume of effector cells (spleen cell populations from each of the nine

groups of mice) were added in triplicate wells to give final target:effector ratios of 10:1, 5:1, 2.5:1, 1.25:1, 0.62:1 and 0.31:1. A non-radioisotopic colourimetric assay employing lactate dehydrogenase (LDH) release to measure CTL activity was used (CytoTox 96® Assay, Promega). This required additional control wells also prepared in triplicate containing: (a) effector cells corresponding to each cell concentration; (b) target cells adjusted to 1×10^5/ml in 100 µl of media; (c) 1×10^5/ml target cells with 10 µl of lysis solution (0.8% v/v Triton-X 100™ in water); (d) 100 µl media with 10 µl lysis solution and (e) 100 µl of media alone. A positive LDH control was also included with the kit. Plates were centrifuged at 250 g for 4 min to allow effector and target cell contact and incubated for 4 h at 37°C in a humidified atmosphere containing 5% CO_2 in air (v/v). After incubation 10 µl of lysis solution was added to each experimental well and to control wells (b) and incubated for a further 45 min at 37°C in a humidified 5% CO_2 in air atmosphere. After incubation 50 µl volumes were transferred from each well, placed into corresponding wells of a flat bottomed 96 well microtitre plate to which a further 50 µl of substrate mix were added and incubated in the dark at room temperature for 30 min. After incubation 50 µl of stop solution were added to each well and the A_{490} of each well was determined using an automatic plate reader (Labsystems). The percentage cytotoxity was determined as below, using the formula supplied with the CytoTox ® kit.

$$\% \text{ Cytotoxicity} = \frac{A_{490}\text{ Experimental} - A_{490}\text{ effector (a)} - A_{490}\text{ target cells (b)}}{A_{490}\text{Lysed target cell control (c)} - A_{490}\text{Media containing lysis solution (d)}} \times 100$$

4.2.9 Measurement of MHC binding by HPV-16 E5 6-mer peptides

The RMA cell line is a subline of the Rauscher virus induced T-cell lymphoma RBL-5 of C57B1/6 mice (H-2^b; [K^b & D^b]), its mutant sub-line RMA-s has a reduced MHC-I cell surface expression (Ljunggren *et al.*, 1985; 1989; Karre *et al.*, 1986; Townsend *et al.*, 1989). RMA-s express approximately 5% of H-2^b cell surface molecules compared to RMA cells. Furthermore, these molecules are unstable at 37°C due to the lack of peptides in the antigen-binding groove. However, they can be stabilised by supplementing culture media with exogenous peptides capable of binding to either of these alleles (Ljunggren *et al.*, 1990; Schuhmacher *et al.*, 1990). The MHC-I binding of a peptide can therefore be quantified indirectly by measuring the expression of cell surface MHC-I (H-2^b) molecules (Townsend *et al.*, 1989; Reinholdsson-Ljunggren *et al.*, 1993; Dillner, 1994). Previous studies have screened synthetic HPV-16 E6 and E7 peptides for the ability to stabilise MHC-I expression on RMA-s cells using this method (Stauss *et al.*, 1992; Feltkamp *et al.*, 1993; Dillner, 1994).

It was not possible to measure peptide binding specific for H-2^d molecules, the Balb/c haplotype, but considering that MHC-I molecules have a broad specificity for antigen this data should provide a general analysis.

RMA and RMA-s cell lines were maintained in RPMI/FBS in a humidified 37°C incubator with 5% CO_2 in air (v/v). Cells were subcultured every 3 days and were grown in 75cm^3 flasks (Corning). When required, 4×10^4 viable RMA-s cells were dispensed into wells of a 96 U-well plate in 40 µl of RPMI/FBS. Individual HPV-16 E5 6-mer peptides were adjusted to 300 µM in 20 µl of culture media and added to each well to give a final concentration of 100 µM per well. Plates were incubated for 16 hours in a humidified 5% CO_2 in air incubator at 37°C and each peptide was assayed in quadruplicates. The assay plate also contained twelve wells of 4×10^4 RMA cells (100%

MHC-I expression) and twelve wells of 4×10^4 RMA-s cells (5% MHC-I expression) to which no peptide was added.

After incubation, plates were washed by adding 180 µl of ice cold DPBS to each well, vortexing for 10 sec followed by centrifugation at 400 g for 2 min (Denley) after which the supernatants were aspirated. The 96 well plate was placed on ice and 25 µl of goat anti mouse H-2 specific antibody (Pharmingen Ltd.), at a dilution of 1:250 (v/v) in PBS, added to each well. The plates were then agitated, incubated on ice for 1 h and washed 3 times in DPBS. Aliquots of 30 µl of rabbit anti-mouse HRP conjugate were added to each well and the plate was incubated for a further 1 h. Wells were washed six times in ice cold DPBS (above) and fixed to prevent cytolysis and release of endogenous cellular peroxidase by adding 50 µl of formaldehyde (diluted 1:100 in DPBS) and incubating on ice for a further 30 min. Wells were washed twice more with 400 µl of DPBS. After this, an aliquot of 50 µl of Sigma Fast™ OPD substrate was added to each well and incubated at room temperature for 30 min after which the A_{490} was determined using an automated micro-titre plate reader (Labsystems). The mean A_{490} of RMA-s cells without peptide was subtracted from the mean absorbance value of RMA cells. The mean A_{490} of RMA-s cells with HPV-16 E5 peptide was expressed as a percentage of the control RMA cells after subtraction of the mean value of RMA-s cells (without peptide).

4.2.10 Statisical Tests

Paired and unpaired students' t-test (ut) and Fishers' exact test (Fet) were used to analyse data.

4.3 RESULTS

4.3.1. Procedure one

Immunisation of mice with non-conjugated E5 (amino acids 1-83) peptide resulted in the production of antisera with low A_{490} values using plates coated with full length E5 peptide (Figure 4.3). Mean A_{490} values of titrated sera from all immunised mice were significantly greater than those of control wells in serum dilutions 1:50 (ut: p=0.046) and 1:200 (ut: p=0.0003). Maximum A_{490} values for HPV-16 E5 peptides were detected at a serum dilution of 1 in 200.

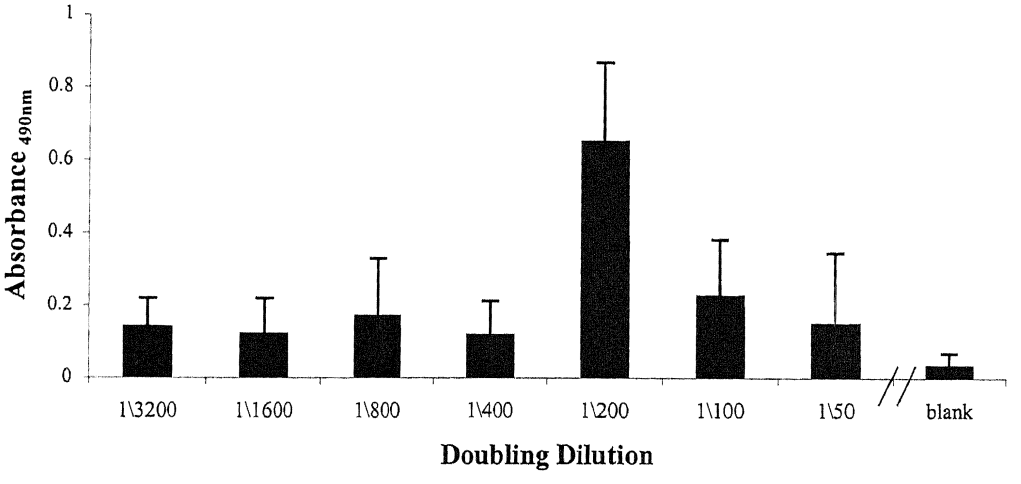


Figure 4.3 Procedure 1: Antisera to whole length HPV-16 E5 in immunised mice. Results are expressed as the mean of nine mice, each tested in triplicate. Error bars represent the standard error of the mean (SEM). Sera diluted in PBS to 1:200 gave the greatest absorbance values, indicating a prozone effect which results when when antigen concentration is low but antibody is relatively abundant (Hyde,1976).

Pepscan™ analyses showed that immunised sera bound to rods 12 (ut: p=0.005), 1 (ut: p=0.04) 11 (ut: p=0.048) 25 (ut: p=0.049) and 26 (ut: p=0.05) and were significantly

higher in sera from all immunised mice compared to that from non-immunised mice (Figure 4.4). Although the peptides on rods 1 and 26 had the lowest hydrophobicity values, immune responses appeared to be independent of hydrophobicity values.

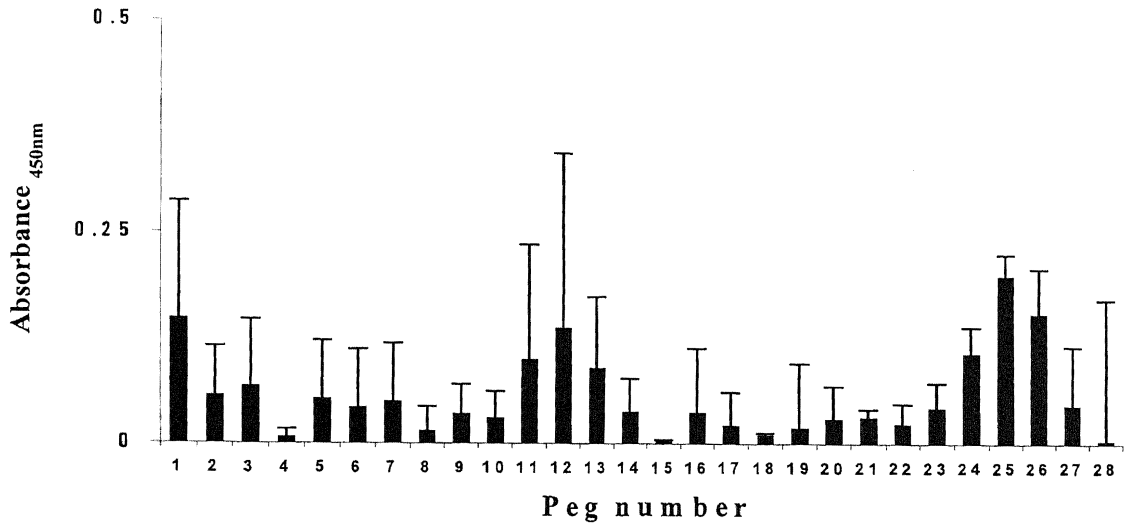


Figure 4.4 Procedure 1: Antisera to HPV-16 E5 5-mers in immunised mice as detected by Pepscans™. Rods numbered 1-27 represent HPV-16 E5 in 5 amino acid sequences, the sequence on each rod is overlapped by 2 amino acids. Results are expressed as serum absorbance values from immunised mice minus serum absorbance values from non-immunised mice. Rod number 28 is the control sequence, made up of randomly selected amino acids (Figure 4.1). Each point represents the mean of nine sera each tested in triplicate. Error bars represent SEM.

Spleens from immunised mice were tested for HPV-16 E5 specific spleen cell proliferation in response to stimulation with HPV-16 E5 6-mers. Five pools of four 9-mer peptides were tested for HPV-16 E5 specific stimulation. For one mouse (mouse 2), there were positive responses to all five pools of peptide, the highest SI was 50.9 and the lowest was 15.5 (Table 4.2). SI from other mice were lower in a second mouse (mouse 7) the

highest value was 2.6, in a third (mouse 8) 2.3 and a fourth mouse (mouse 9) gave a SI of 12.1. Results shown for 5 of 9 mice the remaining mouse spleen populations did not show HPV-16 E5 specific proliferation.

Table 4.2 Procedure 1: Proliferative spleen T-lymphocyte responses in immunised mice to pools of four HPV-16 E5 6-mers compared with EIA data

HPV-16 E5 Specific Stimulation Indices						EIA
Mouse	Amino acids 1-19	Amino acids 18-35	Amino acids 34-51	Amino acids 50-67	Amino acids 66-83	A490 values
2	15.5	16.6	50.9	37.3	23.3	0.7
3	1.2	1.3	1.5	0.8	1.5	0.8
7	1.2	0.6	1.7	2.6	1.0	0.5
8	1.1	1.2	2.3	0.2	1.3	0.4
9	1.0	0.8	0.9	0.8	12.1	0.4

Mouse 1 had the greatest EIA absorbance values at a dilution of 1:200 (1.067) but proliferative spleen cell responses were not detected. The detection of serum antibodies to HPV-16 E5 did not correlate with proliferative spleen cell responses. Non-immunised mice were not tested for HPV-16 E5 specific stimulation.

4.3.2 Procedure two

Sera from all mice used in this procedure were tested against the full length HPV-16 E5 peptide and against ovalbumin which was used as a positive control. Sera were also

tested against PBS and bovine serum albumin which were both negative controls. As only weak HPV-16 E5 specific antibodies were detected in procedure 1, the peptides used in this procedure were conjugated to ovalbumin in order to enhance immunogenicity. Immunisation of mice with ovalbumin conjugated to whole length HPV-16 E5 resulted in the production of antisera that produced low A_{490} values when tested against the full length HPV-16 E5 peptide in EIAs (Figure 4.5). Maximum A_{490} values were detected at a sera dilution of 1 in 200, but reactivity to HPV-16 E5 was not significantly greater when compared with ovalbumin, bsa or PBS. Serum antibody titres to ovalbumin was significantly higher than antibody titres to HPV-16 E5 (pt: p=0.0003), bsa (pt: p=0.0004) and PBS (pt: p=0.0001; Figure 4.5) in two groups of mice immunised with HPV-16 E5 conjugated or mixed with ovalbumin.

Antisera from the same groups, when tested against plates coated with PBS and bsa gave low A_{490} and mean values of titrated sera were not significantly different. Sera from mice immunised with ovalbumin mixed with HPV-16 E5 gave similar A_{490} values in all EIAs (E5, ovalbumin, bsa and PBS coated plates). Serum reactivity to ovalbumin was significantly higher when compared to HPV-16 E5 (pt: p=0.0002), bsa (pt: p=0.004) and PBS (pt: 0.0003). Serum reactivity to HPV-16 E5/ovalbumin conjugates when compared HPV-16 E5/ovalbumin mixtures were not statistically different (pt: all p>0.05).

Sera from all four groups of mice were also tested using Pepscans™ as in procedure one. In mice immunised with HPV-16 E5 conjugated to ovalbumin mean serum reactivity was significantly higher for the amino acid sequence PLLLS (amino acids 31-35) than for any other 5-mer sequence (p=0.04). Otherwise antisera from all four groups gave low A_{405} responses and were not significantly different from each other (pt: all >0.05, data not shown).

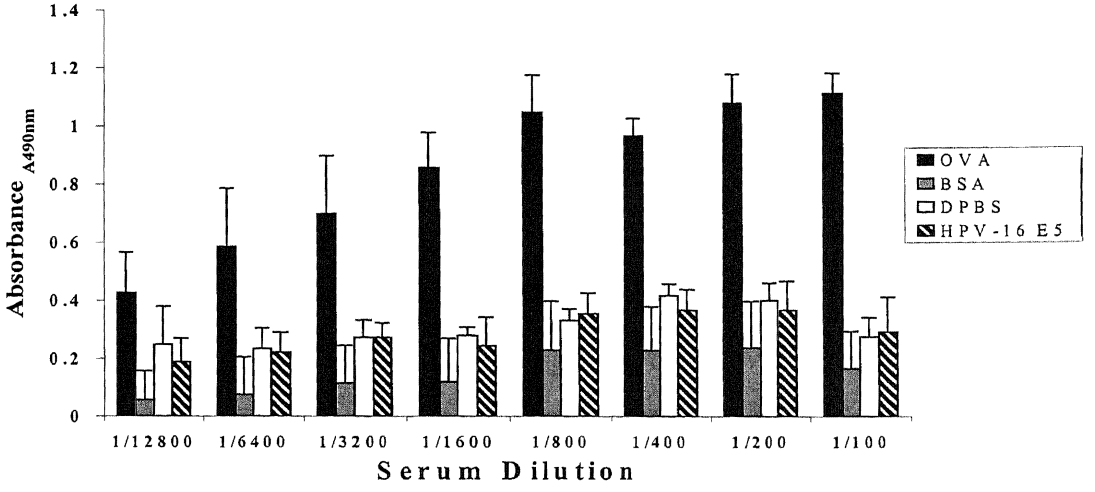


Figure 4.5 Procedure 2: Antiserum to HPV-16 E5, ovalbumin (OVA), phosphate buffered saline (PBS) and bovine serum albumin (BSA) from mice immunised with HPV-16 E5 conjugated to ovalbumin (HPV-16 E5). Greatest absorbance values for antibodies to HPV-16 E5 were detected at serum dilutions of 1:100 & 1:200. Error bars represent SEM.

These data demonstrate the effectiveness of the immunisation procedure in delivering antigen to cells of the immune system as strong ovalbumin specific antibodies were detected. However, antibodies to the HPV-16 E5/ovalbumin conjugate only produced low A_{490} values and it appears that HPV-16 E5 requires further modification in order to improve its immunogenicity. Therefore, in procedure three, HPV-16 E5 alone or conjugated to ovalbumin was incorporated into ISCOMs.

4.3.3 Procedure 3

Serum reactivity to full length HPV-16 E5 was not significantly different between the groups of mice ($p>0.05$). Similarly, when total immunoglobulin was measured in mucous (oral, vaginal of faecal) against full length HPV-16 E5 none was detected in any group or sample (ut=all $p>0.05$).

Mice were immunised with ISCOM preparations to determine whether HPV-16 E5 specific CTL responses could be induced. CTL responses were seen with the highest effector to target ratio of 10 to 1 (Figure 4.6). Cells from mice immunised with whole HPV-16 E5 conjugated to ovalbumin, whole HPV-16 E5 mixed with ovalbumin or just whole HPV-16 E5 had 5.4%, 4.8% and 4.9% cytotoxicity respectively. Mice immunised with 9-mer peptides conjugated to ovalbumin had the greatest cytotoxic activity of 9.8%, 9-mer peptides mixed with ovalbumin had 8.4% cytotoxicity and 9-mers alone also showed cytotoxcity 5.5%. Conjugation of the pooled 9-mer peptides to ovalbumin did significantly enhance CTL responses by almost 2 fold (pt =$P<0.05$).

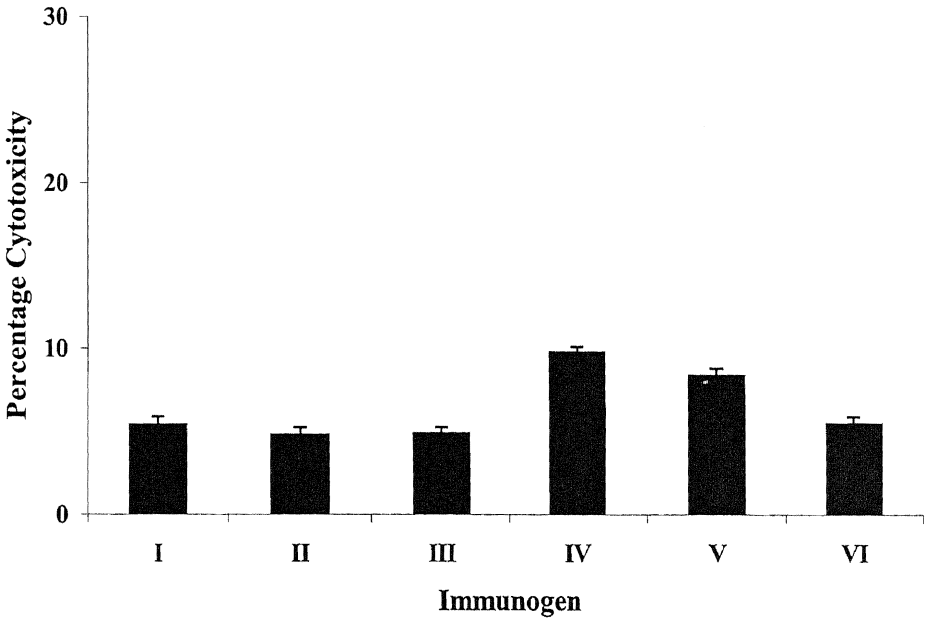


Figure 4.6 Procedure 3: Percentage cytotoxicity to HPV-16 E5 immunogens. **I** = E5 conjugated to ovalbumin; **II** = E5 mixed with ovalbumin; **III** =E5 alone; **IV** = E5 9-mers conjugated to ovalbumin; **V** = 9-mers mixed with ovalbumin; **VI** = HPV-16 E5 9-mers only. Error bars represent SEM.

4.3.4 Peptide binding Assays

Peptides are presented to CTLs on the cell surface by MHC-I molecules and as CTL responses were detected, it was decided to investigate the HPV-16 E5 binding to MHC-I molecules. Of the twenty 6-mer peptides tested, 13 were found to bind H-2^b MHC-I molecules and maintain their cell surface expression on RMA-s cells. Peptides that resulted in the highest MHC expression were peptides; 70-75 (162%), 34-39 (153%), 38-43 (147%), 62-67 (98.7%), 54-59 (87%), 74-79 (85.7%), 78-83 (70%), 30-35 (65%), 10-15 (36.4%), 66-71 (32.5%), 6-11 (22%), 14-19 (16%), 50-55 (14.3%); (Figure 4.7).

Of those peptides with the highest MHC-I binding only one, 54-59 did not contain leucine or isoleucine anchor residues but contained a phenylalanine instead.

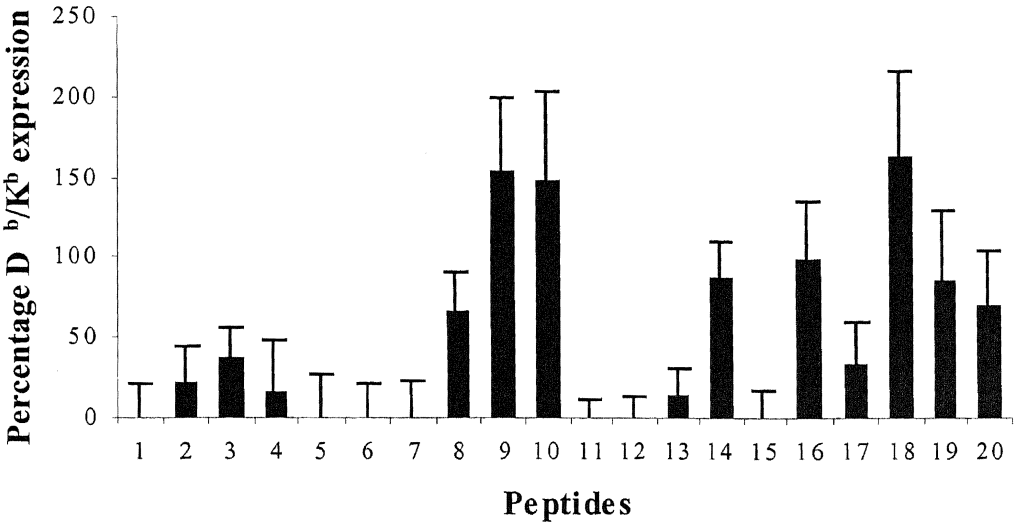


Figure 4.7 Peptide binding assay. This figure shows the percentage H-2^b expression on RMA-s cells after stimulation with HPV-16 E5 6-mers. Error bars represent SEM. Certain regions including the most hydrophobic parts of HPV-16 E5 contain MHC-I binding epitopes.

4.4 Discussion

Data from procedure one show that immunisation with full length HPV-16 E5 alone resulted in the production of low titre serum antibodies. These were mapped to specific HPV-16 E5 epitopes, three of which were highly hydrophobic in nature but two represented the least hydrophobic regions. This suggests that hydrophobicity may not be the only requisite in the selection of immunogenic epitopes.

When HPV-16 E5 was conjugated to ovalbumin in procedure 2, high-titre antibodies specific for HPV-16 E5 were not detected even though high-titre antibodies specific for ovalbumin were. The ovalbumin specific antibodies confirm that the immunisation procedure itself was effective in delivering antigen to the host and suggests that ovalbumin may not be the most suitable carrier for HPV-16 E5. It could be an indication of the poor immunogenicity of HPV-16 E5. The detection of ovalbumin specific antibodies provided a positive control for subsequent immunisations and ovalbumin was included in procedure 3.

Serum and mucosal antibodies reactive with HPV-16 E5 peptides were investigated after procedure 3 but were not detected. The absence of mucosal antibody responses after ISCOM immunisation may be due to the significant dilution involved during the collection of samples. On hindsight absorbant wicks designed to collect mucosal secretions from small animals should have been used and these may have been re-suspended in smaller volumes.

Immunisation with full length HPV-16 E5 alone, resulted in spleen cell proliferation in response to stimulation with pooled peptides. Such a response may be an indication of T-lymphocyte memory, where immunisation with HPV-16 E5 has primed a population of spleen cells which recognise the antigen on a subsequent exposure. Generally, the results in mice, correlate with the general findings in human subjects in Chapters Two and Three,

where strong T-lymphocyte like proliferative PBMC responses were detected in study subjects with the detection of low-titre antibodies. Only two subjects with proliferative PBMCs (Chapter Two) also had concordant antibody responses (Chapter Three). The presence of cellular immunity in the absence of humoral immunity is suggestive of a delayed-type hypersensitivity response, mediated by Th_1 cells.

Peptides incorporated into ISCOMs usually prime MHC-I and induce strong CTL activity (McMowat *et al.*, 1991) and the inclusion of HPV-16 E5 into ISCOMs did result in the generation of CTLs. Although highly toxic, it is possible to immunise with very small quantities of Quil A (the major component of ISCOMs) mixed with antigen. Mice immunised this way were shown to elicit DTH responses and a strong induction of CTLs (McMowat *et al.*, 1991). This approach was tried in what would have been Procedure 4, but mice immunised this way developed lesions (1 cm diameter) at the site of immunisation and had to be sacrificed.

Of the 13 peptides that bound H-2^b molecules, 9 contained leucine and/or isoleucine residues the remaining peptides contained other hydrophobic residues. This indicates that the hydrophobicity of HPV-16 E5 is not responsible for the poor immune responses seen especially as the most hydrophobic regions of HPV-16 E5 have been shown to bind MHC-I molecules.

It is also possible that HPV-16 E5 is available to the immune system but is poorly antigenic, this may be explained by holes in the T-lymphocyte repertoire (Niedermann *et al.*, 1995). Indeed, the prediction of T-lymphocyte epitopes is difficult as only a few peptides that satisfy binding requirements of MHC class-I molecules are found to be immunodominant epitopes (Sijts *et al.*, 1994).

In conclusion immunisation with HPV-16 E5 peptides induces CMI and low-titre antibodies in mice. There is evidence suggesting that HPV-16 E5 is processed and

presented by the MHC-I pathway but may not be efficient. It is evident from the high-titre ovalbumin-specific antibodies detected after Procedure 2 that an alternative route or method of immunisation is required for HPV-16 E5. Previous studies of *in vivo* immunisation with synthetic peptides emulsified in incomplete Freunds adjuvant report strong CTL responses to HPV-16 E6 and E7 proteins (Aichele *et al.*, 1990; Kast *et al.*, 1991). It may therefore, be worth considering alternative adjuvants for HPV-16 E5. A more effective immunisation procedure may result in an effective immune response and a range of adjuvants and delivery systems need to be investigated.

Chapter Five

The Effects of Human Papillomavirus Type-16 E5 peptides and DNA on MHC Expression and Cellular Gene Expression

5.1 INTRODUCTION

Data from Chapters Two, Three and Four indicate that immune responses to HPV-16 E5 occur. These are predominantly cell-mediated responses accompanied by low-titre antibodies. Peptide binding assays in Chapter Four confirm that certain HPV-16 E5 epitopes do bind murine MHC-I molecules and may therefore be presented to cells of the immune system.

Both MHC-I and MHC-II are crucial for the production of an immune response. MHC-I molecules generally present intracellular proteins, these include host self-proteins and proteins produced by intracellular pathogens such as viruses. MHC-II molecules present extracellular proteins that have been phagocyctosed from the extracellular environment. The lack, or the low-level expression, of MHC-I can result in cells that are not recognised by CTLs as infected or neoplastic and may provide a means for cells to escape immune surveillance. Indeed, alterations in MHC-I and MHC-II cell surface expression have previously been described in several human cancers including: malignant melanoma (Ruiter *et al.*, 1986), hepatocellular (Patterson *et al.*, 1988), gastric (Sakai *et al.*, 1987), colorectal (Van den Ingh *et al.*, 1987) and laryngeal (Esteban *et al.*, 1990) cancers. The importance of MHC-II in HPV associated cervical lesions requires investigation but due to shortage of time, it was not possible to investigate MHC-II molecules in this study but MHC-I was investigated. Cell surface expression of MHC-I molecules is often reduced on virally infected cells and in several cases, this effect has been shown to be due to active interference of the MHC-I pathway by the infecting virus (Table 5.1). This obstruction of the normal functions of the MHC-I pathway may be as a result of viral evolution, where evasion of the immune response results. HPV-16, like other viruses may also interfere with the MHC-I pathway, as reduction in MHC-I expression occurs in cervical cancers (Connor & Stern, 1990; Cromme *et al.*, 1993; Hilders *et al.*, 1994).

Table 5.1 Examples of viral escape mechanisms affecting MHC molecules

Virus	Function	Reference
Ad 12 EIA	Down regulation of MHC-I transcription	Schouten *et al.*, 1995
Ad E3 gp19k	Retains MHC-I in ER	Paabo *et al.*, 1983; Burget & Kvist, 1985; Hermiston *et al.*, 1993
Murine CMV m152	Blocks MHC transport	Thale *et al.*, 1995; Ziegler *et al.*, 1997
Murine CMV early gene	Down regulation of MHC-I transcription	Campbell & Slater, 1994
Human CMV US3	Retains MHC-I in ER	Ahn *et al.*, 1996
Human CMV US2, US11	Dislocates MHC-I from ER to cytosol	Wiertz *et al.*, 1996; Jones & Sun, 1997
Human CMV US6	Inhibits TAP mediated peptide transport	Ahn *et al.*, 1997; Hengel *et al.*, 1997
HSV ICP47	Inhibits production and transport of antigenic peptides	York *et al.*, 1994
HIV Nef	Induces endocytic degradation of MHC-I	Schwartz *et al.*, 1996
Poliovirus	Blocks transport of MHC-I from ER	Doedens & Kirkegaard, 1995

The loss of MHC-I expression has been observed frequently in malignant cells and in virally infected cells (Maudsley & Pound, 1991; Ruiz-Cabello *et al.*, 1991). Such a loss would allow these cells to escape from CTL mediated killing and increase their oncogenic potential. In virally infected cells additional post transcriptional mechanisms may result in the retention of MHC-I complexes in the ER, such as binding of heavy chains by the Adenovirus E3 glycoprotein or binding of heavy and or light chains by Cytomegalovirus H301 or indeed other proteins (Andersson *et al.*, 1985; Browne *et al.*, 1991; Del Val *et al.*, 1992; Paabo *et al.*, 1989). The loss of peptide could also result in a reduced MHC-I expression. **ER** endoplasmic reticulum, **HSV** herpes simplex virus, **CMV** cytomegalovirus, **HIV** human immunodeficiency virus, **Ad** adenovirus.

It has been suggested that the expression of HPV-16 proteins may result in reduced cell surface expression of MHC-I leading to abnormal presentation of viral and tumour antigens (Cromme *et al.*, 1993). The loss of MHC-I (and MHC-II) expression is thought to occur at the post-transcriptional level and may be related to the loss of peptide transport due to a reduced expression of transporter associated with transport (TAP-) protein (Connor & Stern, 1990; Glew *et al.*, 1992; Cromme *et al.*, 1994). In addition to cell surface expression, the MHC-I light chain β_2-microglobulin has also been shown to be altered in invasive cervical cancer (Connor & Stern, 1990). It is of interest to determine whether HPV gene expression directly or indirectly accounts for the down-regulation of MHC-I in cervical cancer.

It is possible that specific HPV encoded proteins could be involved, which are expressed at various levels in different lesions. The HPV-16 E5 protein has been shown to alter the acidification of endosomes which may explain the disruption of MHC I and II restricted antigen presentation, cellular transport and cellular communication in infected cells (Straight *et al.*, 1995), this makes it a prime target for investigation of MHC-I functional alteration.

The aims of this Chapter were to investigate the presentation of HPV-15 E5 peptides by MHC-I molecules by cells positive for HPV-16 DNA and to compare this in cells negative for HPV-16 DNA. The cervical cancer derived cell-lines included in this study were the XH1b cell-line, which has been estimated to contain 1 HPV-16 genome copy per cell. The SiHa cell-line, which are estimated to contain 40 copies of HPV-16 per cell and the HeLa cell-line which are estimated to contain 100 copies of the HPV-18 genome per cell. These cell-lines were included to allow comparisons of HPV viral load on antigen presentation. The SiHa and HeLa cell lines will also allow a comparison between HPV-16 and -18 infected cell lines. The spontaneously transformed HaCat cell line and normal

human keratinocytes (NHKC) were included to provide a baseline of 'normal' antigen presentation.

The effect of HPV-16 E5 DNA on intracellular protein expression was also monitored using the ovalbumin gene tagged to a green fluorescent protein (GFP). The Pat-16 (contains the entire HPV-16 genome, Jewers *et al.*, 1992) and Pat-16X (contains the HPV-16 genome with a stop codon in the E5 ORF) plasmids (Dr B. Kell) were used to determine whether HPV-16 E5 DNA had any direct effects on the intracellular expression of ovalbumin. Ovalbumin was chosen as it is known to be highly immunogenic and efficiently expressed and processed through the MHC-I pathway (Moore *et al.*, 1988).

5.2 METHODS

5.2.1 Keratinocyte cell lines

The HPV DNA negative cell lines used were primary normal human keratinocytes (NHKC) isolated from neonatal human foreskins (see below) and the HaCat cell line (provided by Kermani, F.). The HPV DNA positive cells used were; HeLa (HPV-18$^+$; ECACC) SiHa (HPV-16$^+$; ECACC) and XHib (HPV-16$^+$). The HPV DNA status of the negative cell lines was established using PCR as described in section 2.2.3. NHKC (see section 5.2.1.1) were maintained in KMK-2, whilst the HaCat, XH-1, SiHa, Caski and Hela cell lines were all maintained in DMEM media supplemented with 10 % (v/v) FBS, 2 mM l-glutamine, 100,000 U/L benzylpenicillin and 0.1 g/L streptomycin sulphate (DMEM/FBS). All cells were kept at 37°C in a humidified incubator with 5% (v/v) CO_2 in air.

5.2.1.1 Isolation of keratinocytes from newborn human foreskins

Neonatal foreskin from routine circumcision operations (St Thomas' Day Care) was collected into 10 ml of collection media consisting of DMEM, supplemented with 5% (v/v) FBS, containing 100,000 U/L benzylpenicillin, 0.1 g/L streptomycin sulphate and 0.5 g/L amphotericin (Sigma). Tissue was incubated overnight at 4°C then transferred to fresh collection media and dissected with a scalpel into 4 mm strips removing excess adipose tissue and blood. Strips were rinsed with DPBS without antibiotics, placed into 10 ml of dispase II (neutral protease, Boehringer Mannheim) and incubated at 37°C for 3 h. After incubation strips were placed into a sterile petri-dish (Corning) and the dispase II discarded. Using fine sterile forceps the epidermis was pulled away from the dermis. Both portions were placed into a universal containing 5 ml of trypsin EDTA (Sigma), pre-warmed to 37°C and shaken vigorously before being incubated at 37°C for 5 min.

After incubation the universal was shaken once again and the contents passed through a sterile wire mesh to collect a single cell suspension free from dermal and epidermal debri. The resulting cell suspension was washed and re-suspended in DMEM, supplemented with 10% (v/v) FBS, 2 mM l glutamine, 100,000 U/L benzylpenicillin, 0.1 g/L streptomycin sulphate and 0.02 g/L EGF (Sigma, DMEM/FBS). The number of cells were adjusted to 1×10^5/ml and 1 ml aliquots placed in 6 well tissue culture plates (Corning) which were then incubated overnight at 37°C. After incubation the non adherent cells and the serum containing media were removed by aspiration and the remaining adherent cells cultured in 2 ml of keratinocyte serum free media (KMK-2, Sigma).

5.2.2 Interferon-gamma dose response assay

Cytokines secreted by T-lymphocytes, for example, IFN-γ, up-regulate cell surface MHC-I expression. An IFN-γ dose response assay was therefore developed to determine whether cell surface MHC-I expression could be up regulated on the chosen keratinocyte cell lines, thus assessing their suitability for this assay. All cell lines were adjusted to 1×10^5 cells/ml in DMEM/FBS, dispensed as 100 µl volumes into wells of a 96 flat well microtitre plate (Corning) and incubated overnight at 37°C in a humidified incubator with 5% CO_2 in air. The media was then aspirated and replaced by DMEM/FBS supplemented with increasing concentrations of IFN-γ, 0.75, 1.5, 3.1, 6.25, 12.5, 25, 50 & 100 mg/L per well in triplicate. The microtitre plate was then incubated at 37°C in a humidified incubator with 5% CO_2 (v/v) in air for 6 h. The IFN-γ containing media was aspirated and the plate washed six times in ice cold DPBS. The cells were fixed, to prevent cytolysis and subsequent release of endogenous cellular peroxidase, by adding 50 µl of formaldehyde, diluted 1:100 (v/v) in DPBS and incubating on ice for 30 min. Cells were then washed three times in DPBS and 30 µl aliquots of goat anti-human HLA-A,B,C HRP conjugate (Pharmingen), diluted 1:300 in PBS, were added to appropriate wells and the plates incubated at room temperature for 1 h. Plates were washed three times with 400 µl of DPBS then 30 µl of mouse anti goat antibody (Sigma) were added to each well and incubated at room temperature for 1 h. The wells were washed three times with 400 µl of DPBS/well and 100 µl aliquots of Sigma fast OPD substrate were added to each well. The microtitre plate was then incubated in the dark at room temperature for 1 h. The reaction was terminated by the addition of 25 µl of 1 M sulphuric acid and the A_{490} of each well determined using an automated plate reader (Labsystems).

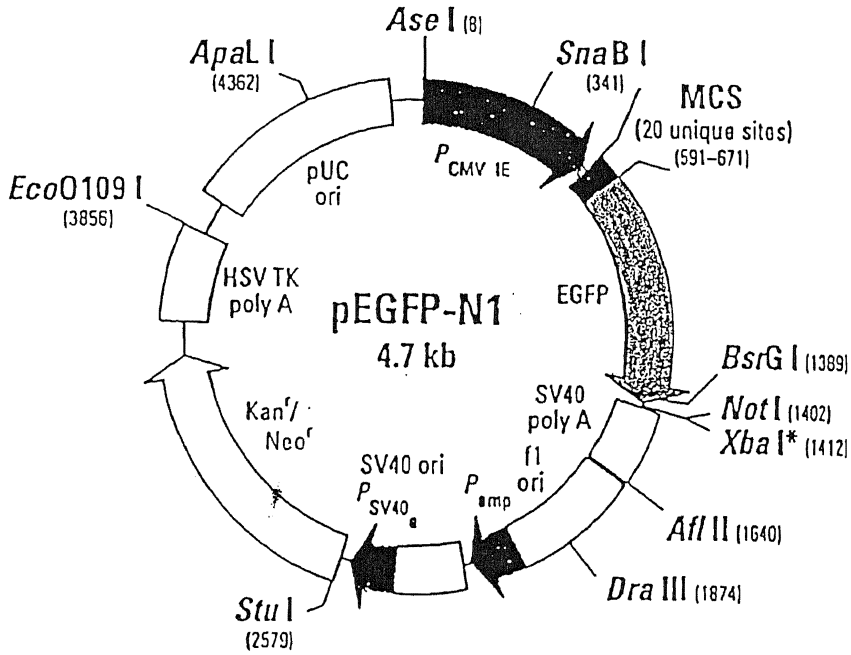
5.2.3 The effects of human papillomavirus type-16 E5 peptides on MHC-I cell surface expression

Pooled HPV-16 E5 9-mer peptides (Figure 2.2) were introduced into the cytoplasm of each cell line (Okada & Rechsteiner, 1982). Briefly, the number of cells were adjusted to 1×10^4/ml and 100 µl aliquots were added to wells of a 96 flat well microtitre plate which was then incubated overnight at 37°C in a humidified CO_2 incubator. The plate was washed once with 200 µl of DPBS. Cells were then re-suspended in 100 µl of a pre-warmed (37°C) hypertonic solution consisting of, 2 mM HPV-16 E5 peptides in 0.5 M sucrose (Sigma), 10% (w/v) polyethylene glycol 1000 (PEG 1000, Sigma), in Hams F12 media (Sigma). Cells were incubated for 10 min at 37°C in a humidified incubator with an atmosphere of 5% (v/v) CO_2 in air. This solution was gently aspirated and replaced with 200 µl of 1 part Hams F12 media to 2 parts (v/v) tissue culture grade water pre-warmed to 37°C and incubated for a further 5 min (see appendix A.5 for macropinocytosis). The plate was incubated overnight at 37°C in a humidified incubator with 5% CO_2 in air after which the cells were stained for cell surface MHC-I expression.

5.2.4 The effect of human papillomavirus type-16 E5 DNA on the cellular expression of ovalbumin

The *pEGFP-N1* plasmid (Clonetics, Figure 5.1) encodes green fluorescent protein (GFP) and an SV40 promoter ensuring a strong green fluorescent signal in transfected cells. The *pEGFP-N1* plasmid also encodes a multiple cloning site (MCS) directly before the GFP sequence allowing the insertion of DNA, this permits the protein product of the inserted DNA sequence to be expressed with a GFP tag. The migration of inserted gene products has been visualised using this technique and was shown not to affect transport of the modified protein (Scales *et al.*, 1997).

The gene encoding ovalbumin has been cloned into the *pAc-neo-ova* gene (Figure 5.2, Moore *et al.*, 1988). The ovalbumin gene was excised from *pAc-neo-ova* and inserted into the *pEGFP-N1* plasmid to allow its expression to be monitored in cells using a fluorescent microscope.



5'A↓AG CTT CGA ATT CTG CAG TCG ACG GTA CCG CGG GCC CGG↓ GAT CCA CCG GTC GCC ACC ATG GTG 3'

HindIII cut site

BamHI cut site

Figure 5.1 The *pEGFP-N1* plasmid and multiple cloning site. Upstream -In order to ensure that the ovalbumin insert remains in frame the HindIII cut site is engineered into the ovalbumin insert which leaves an overhanging A. This releases an overhanging AG which can be ligated to form a new codon and changes nucleotide 20 of ovalbumin from a G to a T. **Downstream** -The downstream primer incorporates a BamH1 cut site ensuring that the ovalbumin insert is kept in frame. The BamH1 cuts after a codon triplet resulting in three changes; bp 1214 from T to G, bp 1216 from T to A & bp 1218 from T to C. Figure reproduced after kind permission from Clontech Laboratories Inc.

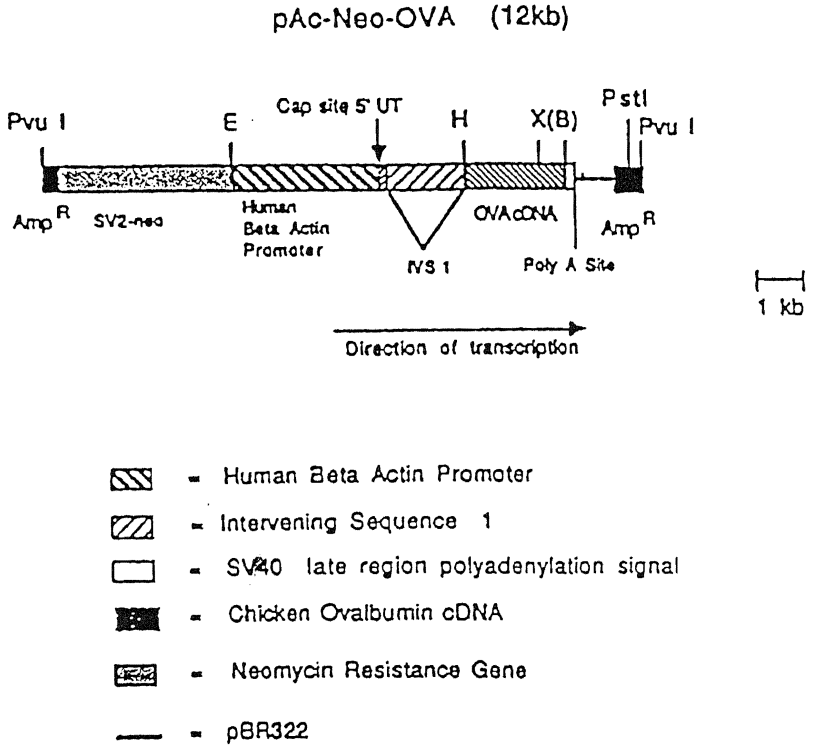


Figure 5.2 The linearised *pAc-Neo-OVA* plasmid. The complete ovalbumin DNA was subcloned into the mammalian pHβApr-1-neo at the BamHI and HindIII sites under control of the human β-actin promoter. *pAc-neo-OVA* also contains the neomycin resistance gene under control of the SV40 promoter. The restriction sites shown on the map are **B**=*BamHI*; **E**=*Eco RI*, **H**=*HindIII*, **X**=*XbaI*. Reproduced after kind permission from Moore *et al.*, 1988.

The published ovalbumin DNA sequence was analysed for enzyme cut sites using a computer program (DNASIS, Hitachi Corporation). Enzyme cut sites not present included *Hind*III and *Bam*HI. These cut sites were present within the MCS of the *pEGFP-N1* plasmid and were considered when designing upstream and downstream

oligonucleotide primers for ovalbumin using a computer program (Oligo, Hitachi). The ovalbumin DNA sequence was removed from the *pAc-neo ova* plasmid by an overnight digestion with 1 unit/μl of the enzyme *EcoR*1 (New England Biolabs), the digested plasmid was electrophoresed through a 1% agarose gel and was purified using a commercial gel extraction kit (Qiagen). The purified ovalbumin DNA sequence was amplified by PCR using oligonucleotide primers that introduced ends complementary for the *pEGFP* plasmid after digestion with *Hind*III and *Bam*HI (New England Biolabs, Table 5.1).

Table 5.2 Ovalbumin oligonucleotide primers

	Ovalbumin primers
Ovalbumin DNA sequence position 11-40 base pairs Upstream Primer	ATC TTT CGA **C**AT AAC GGA AAT CGT GAG TTC TAG AAA GCT **T**TA TTG CCT TTA GCA CTC AAG
Ovalbumin DNA sequence position 1196-1225 base pairs Downstream Primer	AGA GAA GAA ACC GTC TAC **A**C**A** A**A**G GGG AAT TCT CTT CTT TGG CAG ATG **G**G**A** T**C**C CCC TTA

The bp shown in bold and underlined are those changed from the original ovalbumin primer sequences.

In a 1.5 ml microcentrifuge tube, 200 µl of the ovalbumin PCR product, 100 µl of H_2O and 300 µl of phenol:chloroform:isoamyl alcohol (25:24:1, v/v, Sigma) were added together, mixed by vortexing for 10 seconds and then centrifuged for 5 min at 13,000 g. The upper aqueous phase was transferred to a fresh 1.5 ml microcentrifuge tube to which an equal volume (300 µl) of chloroform:isoamyl alcohol (24:1, v/v) was added. The tubes were vortexed briefly and centrifuged for 2 min at 13,000 g. The upper aqueous phase was transferred to a fresh tube and the DNA was precipitated in 1 ml absolute alcohol at -20°C overnight. Tubes containing the precipitated DNA were centrifuged for 30 min at 13,000 g. The pellet of precipitated DNA was then washed with 70% (v/v) aqueous ethanol, dried under vacuum for 10 min and then re-suspended in 20 µl of H_2O.

Both the ovalbumin PCR product and the *pEGFP* plasmid were digested with *Hind*III and *Bam*HI to obtain the correct sequences for ligation as follows. Ovalbumin DNA was ligated to vector using DNA ligase. Briefly, three volumes of ovalbumin DNA were added to one volume of *pEGFP* plasmid contained in a 1.5 ml microcentrifuge tube, to this, 300 µl of absolute ethanol were added. The tube was vortexed for 10 sec and centrifuged for 30 min at 13,000 g. The DNA pellet was washed once with 70% (v/v) aqueous ethanol and dried under vacuum. Once dry, the pellet was re-suspended in the ligation mixture which consisted of, 10 µl of ligation buffer (100 mM Tris-hydrochloride pH7.6; 5 mM magnesium chloride), 40 µl of solution A and 10 µl of solution B. The ligation reaction was then incubated at room temperature for 3 h.

The *pEGFP* plasmid was transformed into electrocompetent *E. coli* (Top 10 cells, Invitrogen) cells to select for inserts containing ovalbumin, as follows. The *pEGFP* plasmid containing the ovalbumin insert was adjusted to 10 µg/µl and 1.5 µl placed into a sterile 1.5 ml microcentrifuge tube which was then left on ice for 5 min. The electrocompetent *E.coli.* were removed from storage at -70°C and allowed to thaw slowly

on ice. Once thawed, an aliquot of 30 µl was added to the pEGFP/ovalbumin plasmid, mixed by pipetting once and left on ice for 1 min. The *E.coli*/plasmid mixture was transferred to a chilled electroporation cuvette (BioRad) and gently shaken to the bottom. Condensation was removed along with any air bubbles and the cuvette placed in the chamber of an electroporater (Gene Pulser, BioRad) and pulsed with 2.5kV (current 25µF, Resistance 200Ω). The cuvette was removed immediately and a 480 µl aliquot of SOC media, consisting of 0.2% tryptone, 0.05% yeast extract, 0.01 M sodium chloride, 0.0025 M potassium chloride, 0.02% magnesium chloride and 0.02 M glucose (Gibco), added and mixed gently by pipetting once. The contents of the cuvette were transferred to a 15 ml centrifuge tube and incubated at 37°C for 1 h in a shaking incubator (Denley).

After incubation, Top 10 cells were centrifuged for 30 sec at 13,000 g to collect cells at the bottom of the tube. An aliquot of 300 µl of SOC media were removed and cells re-suspended in the remaining volume. A 30 µl aliquot of cells was plated on a pre-prepared LB agar plate consisting of, 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride, 1.5% (w/v) agar (Difco) and 100 g/L ampicillin (Sigma), which was then incubated overnight at 37°C. The DNA was extracted and precipitated as above and the plasmid was transfected into NHKC.

5.2.4.1 Transfection of the Pat-16, Pat-16X and pEGFP/ovalbumin plasmids into normal human keratinocytes

For transfection of Pat-16 and Pat-16X DNA into NHKC two solutions were prepared as follows. Solution A, contained 1.5 µg of plasmid DNA in 100 µl serum free RPMI 1640 media (SFM) and solution B contained 12 µl of lipofectin reagent (Gibco) in 100 µl of SFM. Before use both solutions were allowed to stand at room temperature for 45 min. After incubation both solutions were combined, mixed gently and incubated for a further

15 min at room temperature. Cells grown to 60-80% confluency in 25 cm^2 tissue culture flasks were washed twice in SFM which was then replaced with 3 ml of DMEM/FBS without antibiotics. To each flask, an aliquot of 800 µl of the transfectin/DNA mix was added and the solution was mixed by gentle shaking. Cells were incubated for 18 h at 37°C in a humidified incubator with 5% (v/v) CO_2 in air, after which the media was replaced with 3 ml of complete DMEM/FBS and the cells were incubated for a further 72 h. The cells were then washed three times in DPBS and an aliquot of cells were collected using a cell scraper (Corning) and pelleted in a microcentrifuge by centrifugation at 10,000 g for 10 min at room temperature. The cell pellet was subjected to a Proteinase K digestion to obtain DNA, which was assayed for the presence of HPV-16 E5 and E6 DNA (see section 2.2.3 for details of method).

An aliquot of cells was also transfected with the *pEGFP/ovalbumin* plasmid as described above. After the final 72 h incubation an aliquot of cells taken by scraping was assayed for ovalbumin DNA as follows. A hot start PCR was employed using *T.litoralis* (*T.li*) polymerase (Promega), which allows proof-reading and thermostability for the long PCR reaction required for ovalbumin (Perbal, 1988). A 10 µl aliquot of the extracted DNA (from above) was added to the upper mix, which contained 18 µl buffer (50 mM potassium chloride; 10mM TRIS-hydrochloride, pH 9.0; 0.1% Triton®X-100), 30 µl H_2O and 2 µl of *T.li*. The lower mix contained 12 µl buffer, 6 µl magnesium chloride (concentration obtained after magnesium titration), 8 µl dNTPs (final concentration 200 µM), 4 µl of each primer (final concentration 0.5 µM) and 6 µl H_2O. Reaction tubes were placed on a thermocycler programmed for 1 cycle of 94°C for 5 min, 37 cycles of 94°C for 1 min, 58°C for 1 min and 68°C for 1.5 min and 1 cycle of 68°C for 10 min. PCR products were electrophoresed through a 1% agarose gel and photographed (Figure 5.3, as described in section 2.2.3).

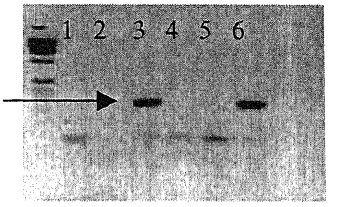


Figure 5.3 Photograph of gel showing ovalbumin PCR product Arrow indicates the 630 bp PCR product. Lanes 1,4=non-transfected HaCat cells, lanes 2,5 non-transfected NHKC, lanes 3,6 transfected NHKCs.

Cells positive for ovalbumin DNA were fixed in a solution of methanol and acetone (1:1, v/v) for 20 min at room temperature then counter stained with 1% Evans blue (Shandon) in PBS (v/v). Once dry the prepared cells were assessed for fluorescence using a fluorescent microscope (Ziess) and the number of fluorescent cells per confluent field were counted using a haemocytometer (Figure 5.4).

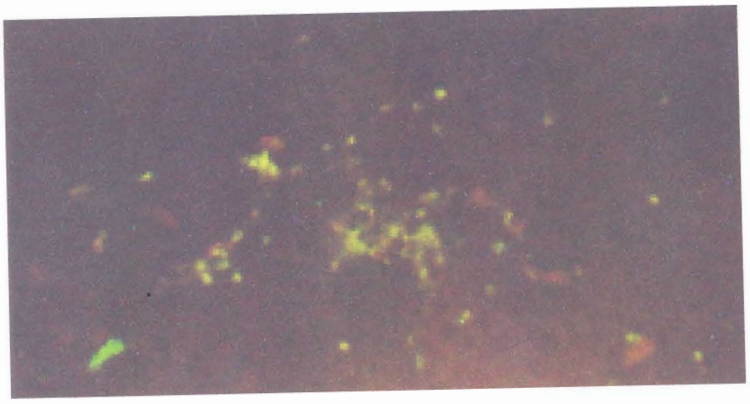


Figure 5.4 Photomicrograph showing transfected keratinocytes. Cells with GFP/ovalbumin appear green due to expression of the ovalbumin/GFP protein. Cells are shown magnified under a ×20 objective.

5.2.5 Statisical tests

Paired students T tests were used to analyse data (pt).

5.3 RESULTS

5.3.1 The cell surface expression of MHC I molecules

The expression of MHC-1 after treatment with IFN-γ was significantly higher in NHKC than compared with HaCat ($p=0.01$), XH1b ($p=0.0002$), SiHa ($p=0.0001$) or HeLa ($p=0.0001$) cell lines (Figure 5.5).

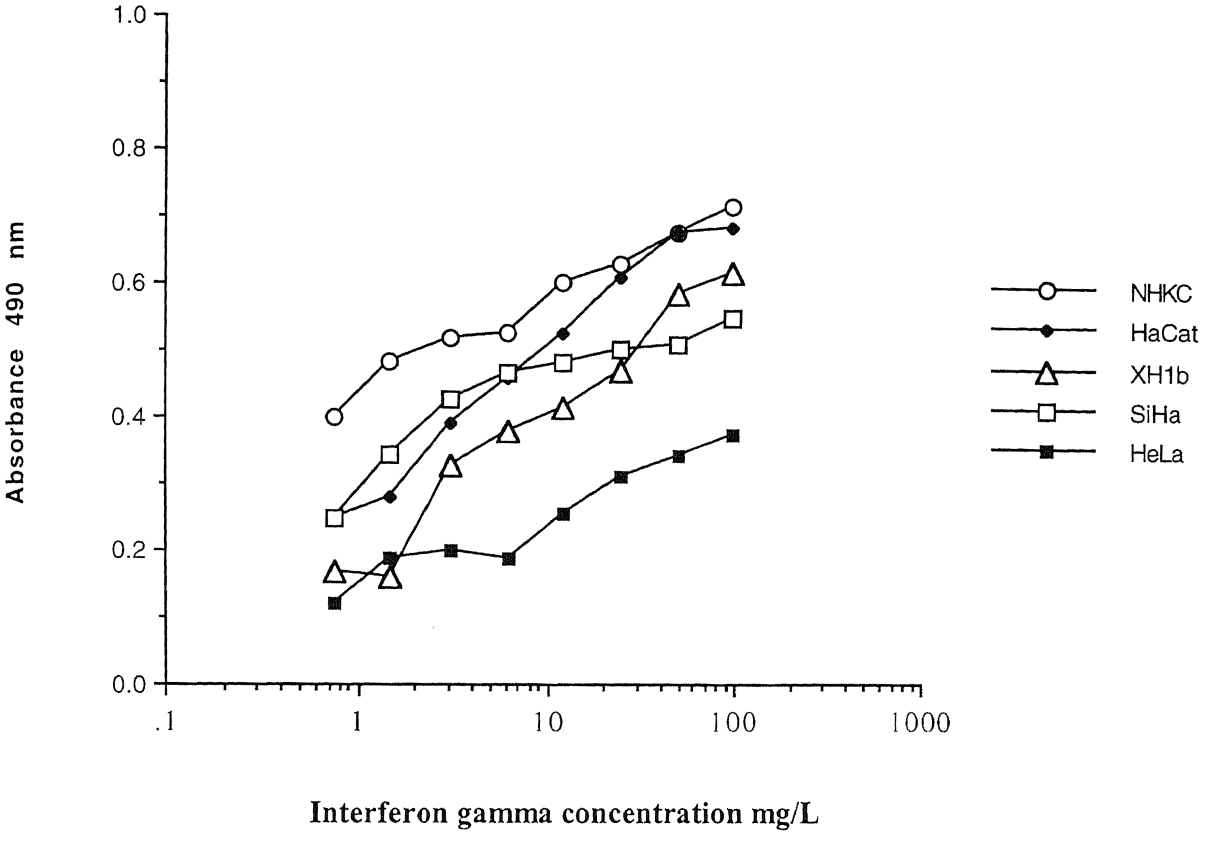


Figure 5.5 Effects of increasing concentrations of interferon-gamma on MHC-I expression. Error bars represent SEM.

When cells were stimulated with pooled HPV-16 E5 9-mers the cell surface expression of MHC-I on NHKC were significantly higher than with INF-γ alone (p=0.05) and also significantly higher than MHC-1 cell surface expression on HaCat (p=0.04), XH1b (p=0.01) and SiHa (p=0.013) and HeLa cells (p=0.023); Figure 5.6). MHC-I expression in HaCat and HeLa cells after stimulation with peptide and IFN-γ compared to IFN-γ alone was not significantly different (p=0.26 & p=0.14 respectively). For XH1b and SiHa cell lines the expression of MHC-1 was significantly higher on cells that were not stimulated with peptide (p=0.002 & p=0.02 respectively).

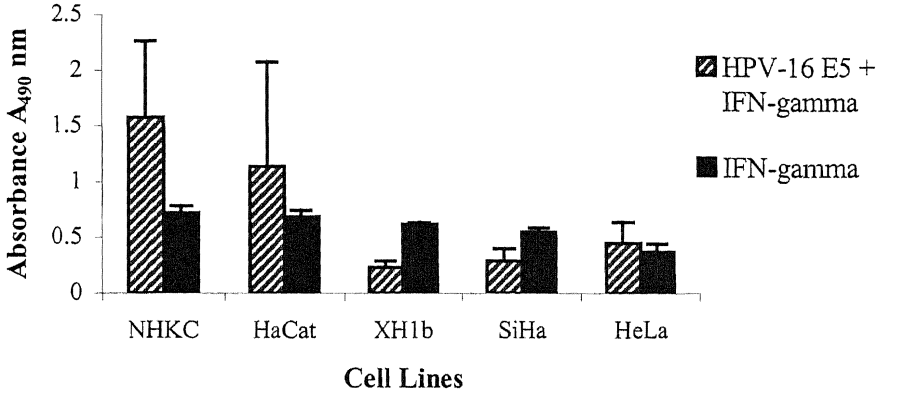


Figure 5.6 Cell surface MHC-I expression on cell lines stimulated with HPV-16 E5 peptides. The mean cell surface MHC-I expression (A_{490}). Error bars represent SEM.

5.3.2 The effects of human papillomavirus type-16 E5 DNA on expression of ovalbumin

All cells transfected with *GFP/OVA* were positive for ovalbumin DNA after PCR. The mean number of fluorescent cells per confluent 10 fields were counted (Figure 5.7).

When analysed, cells that received the *GFP-OVA* construct had significantly more fluorescent cells per field than cells that received both *GFP-OVA* with the *Pat-16X* construct, but not when compared to cells that received the *GFP-OVA* construct with the *Pat-16*. When cells that received both the *GFP-OVA* construct and a Pat construct were analysed, those cells that received the entire *Pat-16* sequence had significantly lower

numbers of fluorescent cells per field that those cells that received *Pat-16X* (Figure 5.7). It was noted however, that the GFP product was not present in the nuclei of cells but was located in the cytoplasm in all cell preparations (Figure 5.4).

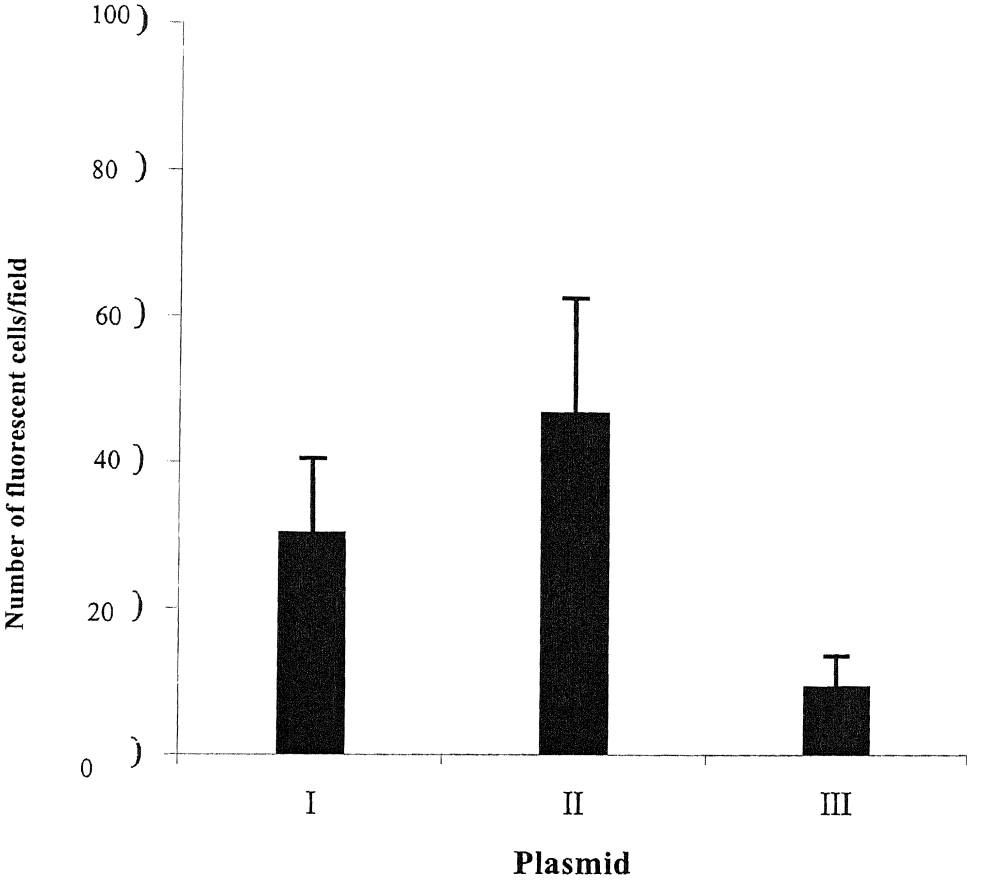


Figure 5.7 Number of fluorescent NHKCs per field A total of 10 fields were examined for fluorescence, the number of fluorescent cells were counted using a tally counter. All fluorescent cells per ×20 field were counted, a total of three fields were counted for each cell preparation. **I GFP/OVA**; **II PAT-16X** HPV-16 DNA without E5 ORF; **III PAT-16** whole HPV-16 genome. Error bars represent SEM.

5.4 DISCUSSION

The low cell surface MHC-I expression that is sometimes seen in cervical lesions may be due to the cells inability to respond to external stimuli. Evidence for this exists in the IFN-γ dose response assay, which showed that NHKC had a higher cell surface MHC-I expression than any other cell line even at low doses of IFN-γ. The HPV DNA negative cell line, HaCat had much lower expression of MHC-I but this expression was equal to that of NHKC at higher doses of IFN-γ. The HPV-18 containing cell line, HeLa had the lowest cell surface MHC-I expression, this cell line also has the highest viral copy number estimated to be 100 viral genomes per cell. The HPV-16 containing XH1b cell line has only one copy of the HPV-16 genome per cell and after stimulation with IFN-γ the cell surface expression of MHC-I was tripled. The cell surface expression of MHC-I on SiHa cells, which have been estimated to contain 40 copies of HPV-16 per cell, doubled after stimulation. It appears therefore, independent of HPV type, either, -16 or -18 that the inability to up-regulate MHC-I expression after stimulation with IFN-γ may result as a consequence of viral load. All cell lines showed elevation of cell surface MHC-I expression after stimulation with IFN-γ.

After stimulation with HPV-16 E5 peptides, those cell lines containing HPV DNA showed no significant changes in MHC-I expression even in the presence of IFN-γ. Indeed, an unusual finding was the significant decrease in MHC-I expression after the addition of peptides in the XH1b and SiHa cell lines. These data suggest that HPV-16 E5 peptides are presented on the cell surface in association with MHC-I molecules in human cell lines and that this expression may be inhibited by the presence of HPV-16 E5 DNA as seen in XH1b, SiHa and HeLa cell lines. It may well be that E5 or indeed, any other HPV-16 or 18 gene product acts to decrease the cell surface expression of MHC-I molecules by interaction with proteins associated with antigen processing (Table 5.1).

The inability to stimulate an increased expression of MHC-I is not limited to cells containing HPV-16 DNA as the HPV-18 containing HeLa cell line also failed to show an increase in MHC-I expression after stimulation. When the effects of HPV-16 E5 DNA were examined in NHKC a significant decrease in intracellular ovalbumin/GFP expression was noted. This may be a result of impaired intracellular communication in E5 infected cells but the mechanisms involved have yet to be determined.

In conclusion, these preliminary results suggest that HPV-16 E5 peptides are presented on MHC-I molecules. However, this presentation may be inhibited by the presence of HPV-16 DNA or protein. It also appears that the expression of HPV-16 E5 DNA disrupts the expression of GFP tagged ovalbumin. If sufficient time had permitted, this would have been investigated further.

Chapter Six

Discussion

The major aim of this thesis was to characterise HPV-16 E5 specific immune responses in women with and without HPV-16 associated cervical disease. The immune system is thought to play an important role in the control of infection as HPV positive lesions are known to regress spontaneously. Indeed, individuals who are immunologically compromised may be up-to 10 times higher at risk for developing HPV associated cervical lesions (Schneider *et al.*, 1983; Rudlinger *et al.*, 1986; Sillman & Sedlis, 1987).

As hypothesised, in Chapter Two, HPV-16 E5 specific CMI was inversely correlated with disease severity and was more frequently detected in women with L-SIL. This suggests that subjects with impaired CMI are more likely to develop cervical disease and less likely to exhibit HPV-16 E5-specific immunity. Those subjects with a fully functional CMI would be less likely to progress to H-SIL or cervical cancer.

HPV-16 E5 DNA sequence variants were investigated as HPV-16 E6 variants are known to alter the conformation of an HLA-B7 binding epitope (Ellis *et al.*, 1995) which may have implications in HPV immunity. Similarly, HPV-16 E5 DNA sequence variants may evade recognition by the immune system as a result of altering possible T or B-lymphocyte epitopes. Three HPV-16 E5 variants were detected in this study population but were not associated with disease severity, or with HPV-16 infection.

It was thought that during the course of natural infection, an effective CMI would result in cell lysis, leading to the production of HPV-16 E5 specific antibodies. However, in Chapter Three, antibodies to full length HPV-16 E5 were not detected and only low-titre antibodies to shorter regions of HPV-16 E5 were seen. It is possible therefore, that only very small amounts of HPV-16 E5 are released from an infected cell after cytolysis, or that HPV-16 E5 remains associated with cellular membranes and is not seen independently of cellular debris. This may result in insufficient amounts of HPV-16 E5 antigen required for

an effective antibody response. Similarly, antibody production may be short lived and become undetectable after antigen is no longer available.

In Chapter Four, when mice were immunised with HPV-16 E5, it was anticipated that HPV-16 E5 specific CMI and antibody responses would result. Strong spleen cell proliferation and a moderate amount of CTL activity was seen but antibody responses were very weak and often only barely significantly higher than controls. This phenomenon may be attributed to the hydrophobic nature of HPV-16 E5 and with its associations with cellular membranes. These data in mice support the data from Chapters Two and Three, where CMI was detected in subjects but antibody titres were low. The peptides used to immunise mice were tested for their ability to bind MHC-I molecules and several HPV-16 E5 9-mer peptides were found to bind and stabilise MHC-I molecules. This indicates that HPV-16 E5 may be presented to CD8+ cells *in vivo* and may result in CMI. Unfortunately, neither proliferative or CTL associated epitopes were mapped in this study. It would also have been interesting to investigate HPV-16 E5 binding to MHC-II molecules had such a model been available. MHC-II restricted CD4+ helper lymphocytes stimulate B-lymphocytes to produce antibody.

In Chapter Five, cells infected with HPV-16 E5 DNA all had reduced expression of cell surface MHC-I molecules when compared to non-HPV infected cells. Similarly cells transfected with the entire HPV-16 genome had a lower level of GFP-tagged ovalbumin expression than cells transfected with GFP-tagged ovalbumin alone or those transfected with the HPV-16 genome minus the E5 ORF. Together, these data indicate that HPV infected cells do not function normally in the context of antigen presentation or protein expression and that virus escape mechanism may be at play.

The development of malignant tumours can be regarded as an example of cells escaping immune surveillance. One possible mechanism is down regulation of MHC-1 expression

affecting recognition and clearance by tumour specific CTLs. The decreased levels of MHC-I are reported in HPV-immortalised cervical cancer cell-lines, this seems to occur at the post transcriptional level as MHC-I mRNAs are often present (Woodworth & Simpson, 1993; Cromme *et al.*, 1993). Similarly, cervical cancers, SIL, cutaneous warts, condylomas and laryngeal papillomas can show down-regulation of MHC-I expression (Connor & Stern, 1990; Cromme *et al.*, 1993; Viac *et al.*, 1990; 1993). The absence of TAP-1 in cervical lesions strongly correlates with the loss of MHC-I expression indicating inhibition of peptide transport by down regulation of TAP-1 as a means of malignant cells to evade immune surveillance (Cromme *et al.*, 1994). Keratinocytes express low levels of MHC-II molecules which may induce tolerance rather than immunity (Chen & Mounts, 1989; Bal *et al.*, 1990).

The interference of MHC-I and TAP has been described for other viruses and it is possible that HPV somehow interferes with TAP expression and transport of antigenic peptides over the ER (Table 5.1). This is supported by the down regulation of MHC-I in HPV positive cell lines under the influence of glucocorticoid hormones which normally in HPV negative cell lines up regulate MHC-I expression (Bartholomew *et al.*, 1997). In addition insertions deletions and point mutations of MHC-I genes are detected in cervical cell lines and their corresponding solid tumour tissue. Tumour phenotypes can be observed that resulted from two or more independent mechanisms that interfere with MHC-I expression (Garrido *et al.*, 1997; Cabello *et al.*, 1998).

It is highly likely considering that as HPVs co-evolved with their hosts that viral escape mechanisms exist. It appears that HPV-16 (and other types) has developed mechanisms, which enables the virus to hide from the immune system. These mechanisms may include: perinatal transmission; absence of cell surface viral proteins; infection of the outermost cells of the host; no viraemic phase; genetic susceptibility to infection/cancer;

the reduced number of Langerhans' cells; immune tolerance or the down regulation of MHC-I *via* TAP-1. However, to survive all this time in the face of immune selective pressure the HPVs needed to develop mechanisms to successfully evade the host immune system. The prevalence of CMI responses against HPV found in the presence of persisting HPV infections and H-SIL suggests that such evasive strategies are at work. Other possible immune evasion mechanisms that may be used by genital HPVs are summarised in Table 6.1.

Conclusions drawn from this study are that women infected with HPV-16 E5 DNA show specific PBMC proliferation which is inversely correlated with disease severity. Humoral immunity exists in asyptomatic women and those with L-SIL indicating that immunity may be impaired in women with H-SIL and cervical cancer.

This thesis also supports the hypothesis that women with intact immune responses are able to combat HPV infection as those who were HPV negative had reactive serum antibodies to HPV-16 E5 9-mers.

It can also be concluded that full length HPV-16 E5 and 9-mers are poor immunogens even after conjugation to immunogenic carriers and incorporation into ISCOMs. However, as some CTL activity was seen in immunised mice, the method of immunisation may need revisions. HPV-16 E5 peptides do show binding to and presentation by MHC-I molecules. It appears that HPV-16 E5 DNA may impair cellular function in some way.

Table 6.1 Possible viral escape mechanisms used by HPVs

Mechanism	Factors	References
Avoidance of host immune system	Supra-basal expression	Stanley, 1994; Stoler *et al.*, 1992; Durst *et al.*, 1992
	Depletion of Langerhan cells	Tay *et al.*, 1987; Viac *et al.*, 1990; Lehtinen *et al.*, 1993; Morelli *et al.*, 1992
	Sequence variation	Ellis *et al.*, 1995; Xi *et al.*, 1997
Altering susceptibility to immune response	MHC-I and Tap down regulation	Cromme *et al.*, 1993; 1994a, b; Keating *et al.*, 1995; Duggan-Keen *et al.*, 1996;
	Apoptosis resistance (bcl-, serpins)	Ter Harmsel *et al.*, 1996; Muller & Tschopp, 1994
	Induction of apoptosis in CTL by interaction with Fas ligand on keratinocytes	Cabello & Garrido, 1998
	Induction of KIR expression on infiltrated CTL by IL-10 & TGF-β	Bertone *et al.*, 1999; Mingari *et al.*, 1998
Disarming of T-cells	T-cell receptor CD-3ζ down regulation	Finke *et al.*, 1993; Matsuda *et al.* 1995; Nakagomi *et al.*, 1993; Kiessling *et al.*, 1996; Levey & Srivastava, 1996; Mulder *et al.*, 1997; Kono *et al.*, 1996; Reichert *et al.*, 1999; de Gruiji *et al.*, 1999
Tolerance, induction and Suppression of cell mediated immune response	MHC-II expression on keratinocytes without co-stimulatory molecules results in un-professional antigen presentation:	Cromme *et al.*, 1993; Coleman & Stanley, 1994; Glew *et al.*, 1992
	-Th1/Th2 balance shift	Wu & Kurman, 1997; Clerici *et al.*, 1997; de Gruijl *et al.*, 1999; Frazer, 1996; Nickoloff *et al.*, 1995
	-Tolerance induction	Doan *et al.*, 1999
	-Immunosuppresive cytokines IL-10 & TGF-β	Majewski *et al.*, 1996

Adapted from Bontkes *et al.*, 1999.

Future work

Considering that CMI to HPV-16 E5 was inversely correlated with disease severity, it would be of value to investigate the effects of treatment on immunity in longitudinal studies. Treatment of cervical lesions could be correlated with HPV-16 infection and immunity over a number of years. Similarly, immune responses to other HPV-16 early proteins (E1, E1-E4 and E2) may also be investigated.

The long-term variation of HPV-16 E5 within individuals or populations over time remains undetermined and the investigation of HPV-16 E5 variants is required. It is possible that HPV-16 establishes a persistent infection in which a single variant predominates and co-infections with additional HPV-16 variants may result in minor populations of HPV-16 genomes. This as with HPV-16 E6 may affect the nature of any immune response or the severity of cervical disease. It is also necessary to correlate HPV-16 E5 variation with severity of disease, ethnicity and other co-factors.

It will also be of interest to investigate the effects of an alternative immunisation procedure in an animal model. Such procedures may include 'DNA vaccination' or use of an L1/E5 chimera.

It is thought that HPV-16 E5 may impair normal cellular function affecting antigen processing and presentation by MHC-I. The HPV-16 E5 protein is an ideal candidate for the investigation of viral escape mechanisms and its site of action could occur at the ER, Golgi or at the plasma membrane. It is also possible that E5 or other HPV early proteins bind to cellular proteins associated with antigen processing located in the ER where there is opportunity for HPV-16 E5 to interfere with their normal functions. The ER is also central to the antigen recognition capacity of lymphocytes because it controls the folding and assembly of antigen receptors and may be the cellular organelle, where HPV-16 E5 exerts

its affect on normal MHC pathways. The work from Chapter Five remains incomplete and requires additional studies in order to clarify the effect of HPV-16 E5 on cellular function and antigen presentation.

Appendix

Appendix 1 Trypan blue dye exclusion test

The trypan blue method of cell viability is based on the principle that live cells do not take up certain dyes but dead cells do. A 4% solution of trypan blue was prepared in PBS (w/v) and 200 µl were added to a 1×10^5/ml cell suspension in a volume of 200 µl. The cells were allowed to stand for 5 min and then transferred to the chambers of a Neubauer haemocytometer. All the cells in the 1 mm centre square and four 1 mm corner squares were counted, a separate count of viable and non- viable cells was recorded. The percentage of cells excluding dye was determined by brightfield illumination using a Nikon microscope (× 10 objective lens).

Appendix 2 The MTT assay

A water soluble yellow terazolium salt MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes (Slater *et al.*, 1963). The formazan can be solubilised using dimethyl sulphoxide and measured spectrophotometrically allowing the measurement of concentration of converted dye. The MTT assay was used to detect cell proliferation as it produces results equivalent to conventional ^{3}H-thymidine incorporation tests but is simpler to use and does not require the use of radioisotopes. This assay has been published for use in several studies including those of interleukin-2 production (Mossman, 1983; Denizot & Lang, 1986).

Appendix 3 Hydrophobicity values of HPV-16 E5 peptides used in this thesis

Amino acids	Hydrophobicity
Full length	
1-83	-1.13
5-mer	
1-5	-0.06
4-8	0.12
7-11	-0.4
10-14	-0.78
13-17	-1.52
16-20	-1.62
19-23	-1.72
22-26	-1.42
25-29	-1.58
28-32	-0.48
31-35	-1.02
34-38	-0.62
37-41	-0.5
40-44	-1.1
43-47	-1.74
46-50	-2.06
49-53	-1.58
52-56	-0.34
55-59	-0.14
58-62	-0.7
61-65	-1.84
64-68	-1.98
67-71	-1.48
70-74	-1.58
73-77	-1
76-80	-0.32
79-83	-0.82

Amino acids	Hydrophobicity
6-mer	
1-7	-0.17
6-11	-0.4
10-15	-0.62
14-19	-1.77
18-23	-1.6
22-27	-1.48
26-31	-0.57
30-35	-0.35
34-39	-0.9
38-43	-1.07
42-47	-1.75
46-51	-2.02
50-55	-1.05
54-59	-0.2
58-63	-1.02
62-67	-1.9
66-71	-1.65
70-75	-1.4
74-79	-0.68
78-83	-0.77
9-mer	
1-8	-0.11
5-13	-0.1
10-18	-1.2
15-23	-1.74
20-28	-1.47
25-33	-0.94
30-38	-0.41
35-43	-0.81
40-48	-1.38
45-53	-1.56
50-58	-0.7
55-63	-0.98
60-68	-1.96
65-73	-1.78
70-78	-1.09
75-83	-0.67

Appendix 4 Measurement of HPV-16 E5 specific PBMC proliferation in Laboratory donors

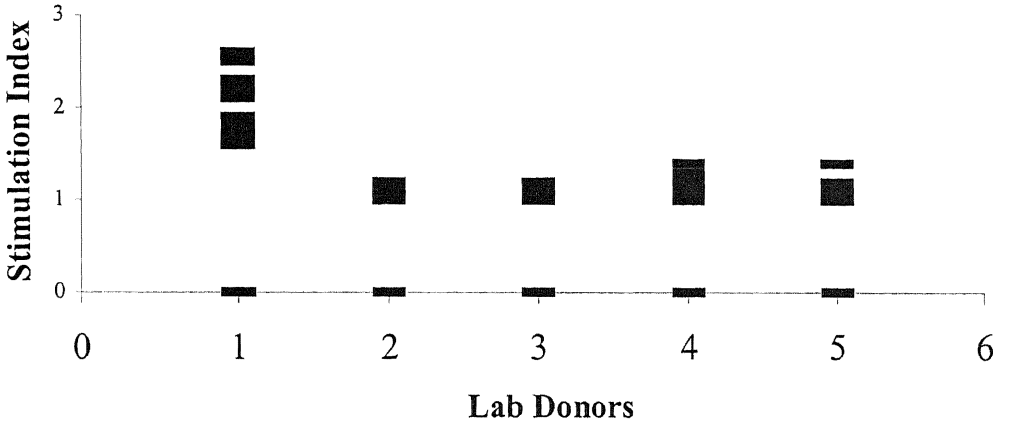


Figure A.1 PBMC proliferation to HPV-16 E5 9-mer peptides in anonymous laboratory volunteers. When tested for HPV-16 E5 specific proliferation only one donor showed SI values in excess of 1.5.

Appendix 5 Macropinocytosis

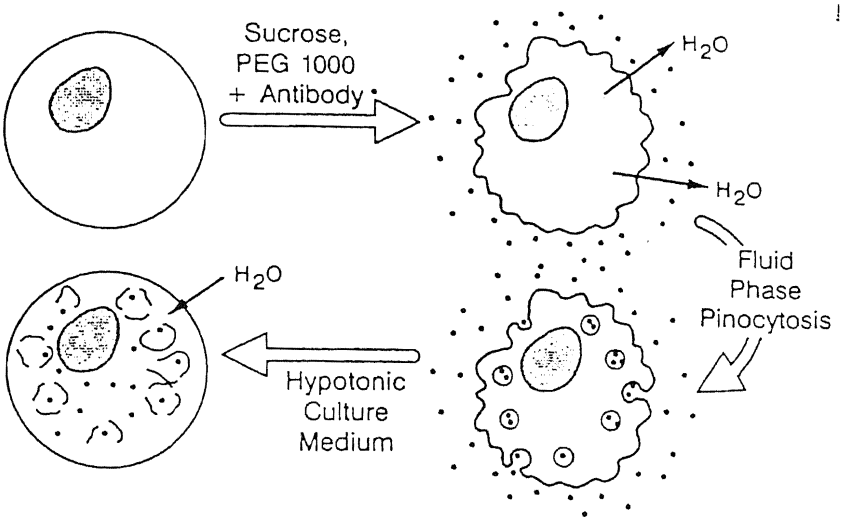


Figure A.2 The principle of macropinocytosis Adapted from Morgan & Roth, 1988. A peptide is included into the MHC-I processing pathway if it is present in the cytosol, *e.g.* proteins (*i.e.* influenza nucleoprotein or ovalbumin) added to the culture media of cells expressing both MHC-I & MHC-II are internalised and processed in association with MHC-II but, when the genes encoding protein are transfected into the same cells so that protein is synthesised endogenously then MHC-I association is seen. Similarly, if these proteins are introduced into the cytoplasm by transiently permeablising the cell membrane using osmotic shock (macropinocytosis) they are also processed associated with MHC-I (Okada & Rechsteiner, 1982).
Macropinocytosis involves two consecutive incubations at 37°C, the first 10 min in hypertonic solution containing HPV-16 E5 peptides and the second 5 min in mildly hypotonic culture medium. The black symbols • represent HPV-16 E5 peptides. This method has little effect on cell viability (Morgan & Roth, 1988).

Appendix 6 MTT assay to evaluate the effect of macropinocytosis on cell viability

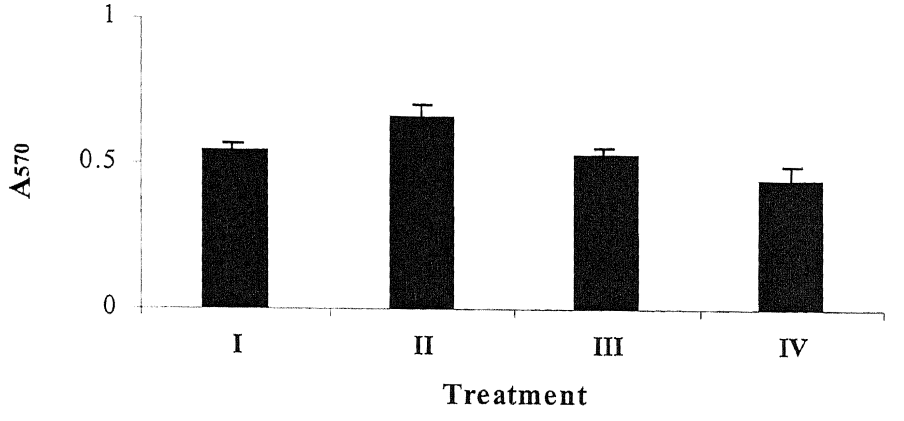


Figure A.3 **I** hypertonic solution only; **II** hypotonic solution only, **III** macropinocytosis, both solutions; **IV** cell culture media only. The above figure shows that macropinocytosis or treatment with hyper or hypotonic solutions does not affect cell viability. Error bars represent SEM.

Appendix 7 pGEM®-T Plasmid

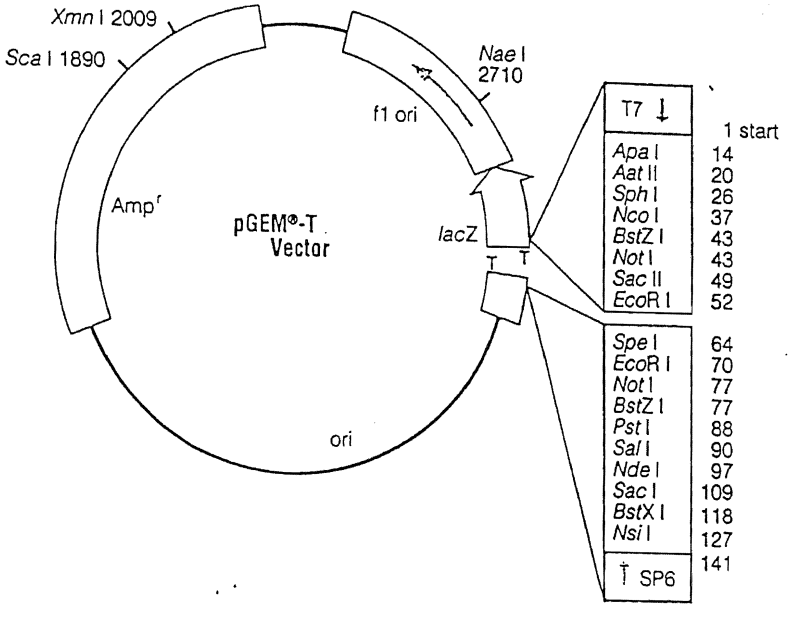
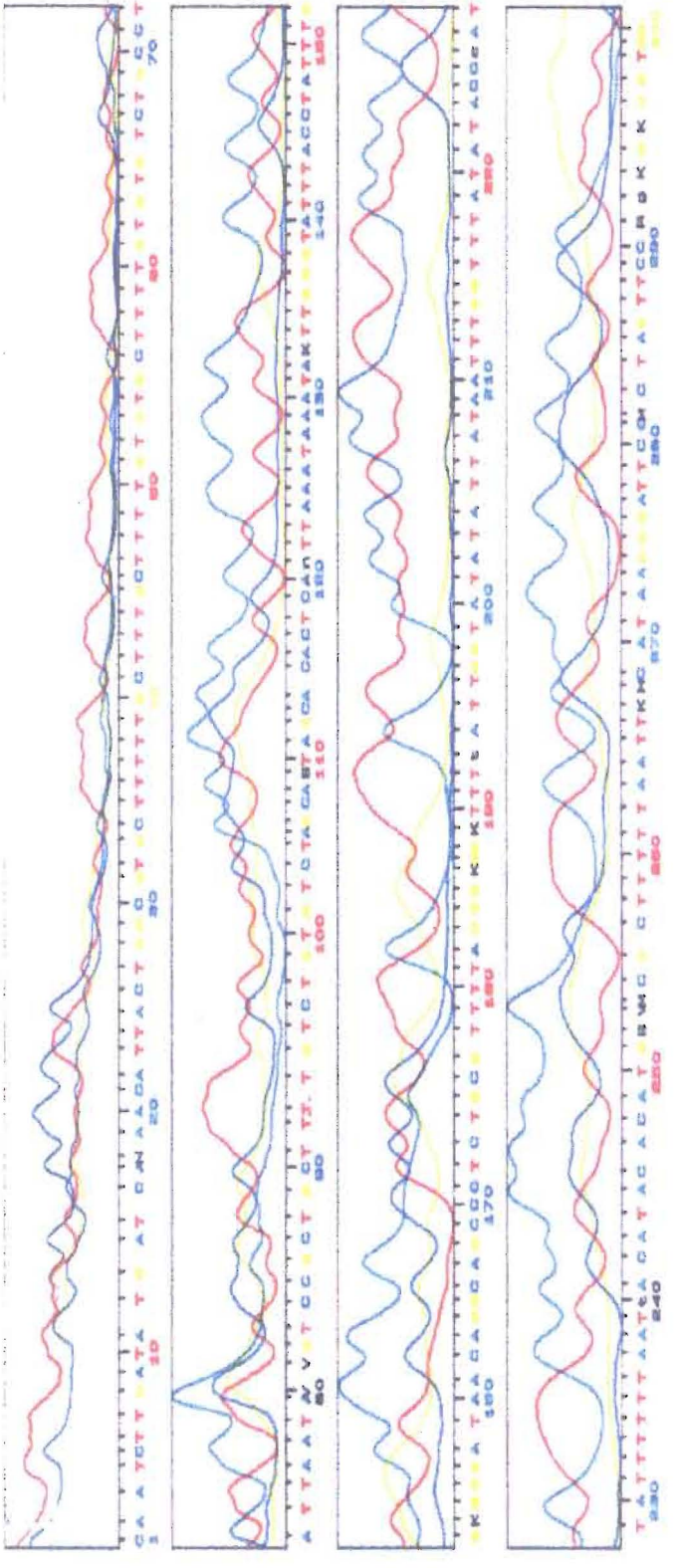


Figure A.4 pGEM®-T Vector. Reproduced with kind permission from Promega.



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Corrigenda