Down-regulation of MHC class I by papillomavirus E5 proteins

El Hadi Saad Mohamed Araibi

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Dedication

To my wife, Jamila, and my son and daughter, Sirag and Sara. I have taken so much of the time that should have been ours together, and you have given without complaint. For this reason and others I consider this thesis as much yours as it is mine. Unfortunately it falls short of repaying the great debt that I owe you all. I love you all.

Author's Declaration

I hereby declare that the work presented in this thesis is original and was performed solely by the author, except where assistance of others is acknowledged. I also hereby certify that no part of this thesis has been submitted in any form to any other universities for the award of a degree.

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ABBREVIATIONS

ATP	Adenosine triphosphate
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cells
AR	Antigen retrieval
ATL	adult T-cell leukemia
β_2M	β_2-microglobulin
BL	Burkitt's lymphoma
BoLA	bovine leucocyte antigen
bp	base pairs
BPV	bovine papilomavirus
BSA	Bovine serum albumin
CDK	cyclin-dependent kinase
CIAP	calf intestinal alkaline phosphatase
CIN	Cervical intraepithelial neoplasia
cm	Centimeters

CR	conserved regions
CTL	cytotoxic T lymphocyte
DAB	diaminobenzidine
DCs	dandritic cells
DD	death domain
DEPC	Diethyl Pyrocarbonate
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	3' deoxyribonucleoside 5' triphosphate
DR	death receptor
E2F	DNA-binding transcription factor
E.coli	Escherichia Coli
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetra-acetic acid
EGF-R	epidermal growth factor receptor
EtBr	Ethidium bromide
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal calf serum
FITC	Flurorescein-isothiocynate
G418	Geneticin, G418-sulphate
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GFP	Green Fluorescent Protein
GTP	Guanosine triphosphate
HaCaT	Human immortalised keratinocyte stable cell line
HBV	Hepatitis B virus
HCC	hepatocellular carcinoma
HCMV	Human cytomegalovirus

HEPES	N-[2-Hyroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HHV8	human herpesvirus 8
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPV	Human papillomavirus
HRP	Horseradish Peroxidase
HSV	Herpes Simplex Virus
HTLV-1	Human T cell leukaemia virus-1
Ig	immunoglobulin
IP	Immunoprecipitation
kbp	Kilo-base pairs
kDa	KiloDalton
KS	Kaposi's sarcoma
l	litre
LANA	latency-associated nuclear antigen
LCR	Long control region
mAb	Monoclonal antibody
MAP	mitogen activated protein
MHC	major histocompatibility complex
ml	millilitre
mM	Millimolar
mRNA	messenger ribonucleic acid
NK	natural killer
NPC	nasopharyngeal carcinoma
°C	degree centigrade
OD	Optical density (light absorbance)
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PSI	pounds per square inch
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline plus 0.1% Tween
PCR	Polymerase chain reaction
PDGF-R	platelet-derived growth factor receptor

PV	Papillomavirusc
Rb	retinoblastoma
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	revolutions per minute
RSV	Rous sarcoma virus
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulphate
STWS	Scott's tap water substitute
SV40	Simian virus 40
TAP	transporter associated with antigen processing
TNF	tumour necrosis factor
TRAIL	TNF related apoptosis-inducing factor
Tris	Tris (hydroxymethyl) aminomethane
Tween 20	Polyoxyethylene sorbitan monolaurate
UV	Ultraviolet
V	Volts
VEGF	vascular endothelial growth factor
v/v	Volume per unit volume
wt	Wild type
µg	microgram
µl	microlitre

Abstract

Like many viruses, papillomaviruses are totally dependent upon host biosynthetic machinery for replicating their genomes and establishing infection. These processes are associated with the production of antigenic proteins that make the virus vulnerable to immune surveillance which ultimately results in elimination of viral infection. However, papillomaviruses have developed mechanisms to escape the cellular immune recognition of the virally-infected cells by encoding proteins that interfere with antigen presentation to immune cells.

Papillomavirus E5 are small oncoproteins of 42 to 83 amino acids in BPV-4 and HPV-16 respectively, expressed at low levels mainly in the deep layers of infected epithelia and down-regulate the expression and transport to the cell surface of the major histocompatibility complex class I (MHC I). The BPV-4 E5 protein inhibits transcription of heavy chain, retains MHC class I in the Golgi apparatus and prevents its transport to the cell surface. In this study, we investigated expression of MHC class I in natural infection by BPV-4. We determined that MHC I is not expressed in cells expressing BPV-4E5. We also determined that expression of BPV-4E5 is accompanied by expression of the proliferation marker (Ki67) also in the differentiated cells of upper layers of papillomas. Down-regulation of MHC class I in papilloma cells expressing E5 would allow escaping recognition by cytotoxic T lymphocytes (CTL). Cells lacking MHC I are subjected to NK cells attack, which recognise and destroy cells lacking surface expression of MHC I unless non classical MHC class I molecules are presented on the cell surface. Because of the impossibility to investigate expression of non classical MHC class I in cells expressing classical MHC class I due to the absence of appropriate antibodies, we investigated the effect of BPV-4E5 on expression of bovine

classical (N*01301) or non classical (N*50001) MHC class I in transfected mouse mastocytoma cells. We determined that E5 does not retain N*50001 MHC class I complex in the Golgi, and does not inhibit the transport of the complex to the cell surface. We also determined that E5 induces degradation of N*01301 heavy chain but does not affect the stability of N*50001 heavy chain. We also determined that retention of MHC class I in the Golgi and thus prevention of its transport to the cell surface requires the C-terminus domain of E5. We also investigated the relationship between HPV-16E5 and HLA class I. HPV-16E5 down-regulates surface expression of HLA-A, but not of HLA-C/E. Because of a lack of antibodies capable of distinguishing C and E, we introduced either HLA-A2 or HLA-E cDNA in mouse mastocytoma cells expressing HPV-16E5. We confirmed that HPV-16E5 down-regulates HLA-A2 by retaining it in the Golgi and inhibits its transport to the cell surface but it does not affect HLA-E. We extended our observations to investigate the effect of HPV-16E5 on expression of MHC class I in cervical intraepithelial neoplasia grade I (CIN I). We determined that in some CIN cells, expression of E5 and HLA class I was incompatible, while in other cases E5 and HLA class I were co-expressed. Expression of E5 in some cells was accompanied by expression of Ki67. We also determined that down-regulation of HLA class I by E5 is independent of expression of HPV-16E7 in raft cultures of HaCaT cells expressing HPV-16E5 only. E5 does not affect expression of HLA-E. Therefore down-regulation of classical MHC class I by PVE5 proteins on the surface of infected cells would allow escaping recognition by CTL while, undisturbed expression of non-classical MHC I on the cell surface would escape destruction by NK cells. It remains to be determined if E5 expressing cells do avoid being killed by both CTL and NK cells.

Chapter One

Introduction

1 Introduction

1.1 Cancer

Cancer is a family of diseases characterized by uncontrolled cell proliferation. The development of cancer is a multistage process in which cells gradually become malignant through a progressive series of alterations. The process of carcinogenesis may be divided into at least three stages: initiation, promotion, and progression based on evidence from experimental models (Dragan et al 1993). The first step of the process, initiation, generally results from an irreversible genetic alteration, one or more simple mutations and or small deletions in cellular DNA within a single cell, leading to cell proliferation. The cells at this stage, although altered at the DNA level, are phenotypically normal. The second stage, promotion, is reversible and does not involve changes in the structure of DNA. The initiated cells undergo selective clonal expansion to form primary tumour (premalignant lesions) characterized by sustained cellular proliferation. During irreversible progression, further genetic and epigenetic changes take place such as DNA methylation which inhibits the transcription of tumour suppressor genes. DNA methylation occurs in the promoter region of tumour suppressor genes, blocking transcription by preventing transcriptions factors from binding to these genes (Jones and Laird 1999). The epigenetic changes affect gene expression, and contribute to irreversible phenotypic changes with highly invasive growth and tendency to metastasize (Sugimura 1992; Vogelstein and Kinzler 1993). Induction of DNA damage and genetic instability has been considered in some cancers to be promoted by environmental exposure to extrinsic agents such as chemicals, radiation and viruses. Extrinsic agents may act in several ways, including the

direct promotion of cell proliferation or interference with apoptosis (e.g, through oncogenic viruses),(Sieber et al 2005).

1.1.1 Oncogenes and tumour suppressor genes

Cancer generally develops after a prolonged latent period which suggests that more than one event must occur within a single cell before manifestation of malignant transformation. Genetic disturbances of two broad classes of genes are required for cancer development and progression.

1.1.1.1 Oncogenes

One class is the proto-oncogenes; these are normal cellular genes. The products thereof act as elements of the signaling pathways that regulate cell proliferation and survival in response to growth factor stimulation. Proto-oncogenes were identified experimentally by the homology of their nucleotide sequences to retroviral oncogenes. The viral and cellular oncogenes have defined a large group of genes (about 100 in total) that can contribute to the abnormal behavior of malignant cells. Proto-oncogenes have been classified according to the functions of their proteins. They include polypeptide growth factors, growth factor receptors, elements of intracellular signaling pathways, and transcription factors. Viral oncogenes were first defined in Rous Sarcoma virus and tumour cell filtrate was able to induce cancer within two weeks after inoculation in healthy chicken (Rous 1979b). The RSV oncogene, termed v-src oncogene, was first postulated by Huebner and Todaro (1969) as a determinant of cancer. Later it was discovered (Stehelin et al 1976) that a cellular homologue of v-*src* is present in normal DNA, which is called c-*src* proto-oncogene. The c-*src* is the prototype of a family of at

least 8 closely related genes encoding protein tyrosine kinases that form a cytoplasmic signal transducer group, one of five groups in which protooncogenes are classified according to the function of their gene products. The signal transducer protein c-src is expressed at a low level in many cell types, also expressed at a high level in certain cells that are specialized for regulated secretion, including neurons, endocrine cells (Brugge et al 1986). The src protein is an essential mediator of the transmission of signals between cell surface and cytoplasm. Src activity initiates a signal transduction cascade, which culminates in the transcriptional activation of the transcription factor Myc and consequently regulates proliferation, adhesion and migration (Brown 1997). In transformed cells, v-*src* suppresses expression of the cyclin-dependent kinase inhibitor p27, leading to rapid transit of the G1 phase of the cell cycle and failure to enter the quiescent state (Johnson et al 1998). Strongly transforming Src proteins lead to loss of actin organisation and disruption of cellular adhesions resulting in cell detachment (Fincham et al 1999)

C-myc is a nuclear phosphoprotein. The *myc* oncogene was originally isolated from the avian myelocytomatosis virus. Protooncogene c-*myc* has been detected in high copy number in lymphomas in humans and mice. Amplification of c-*myc* is due to translocation of an active cellular promoter from the immunoglobulin gene to another chromosome that contains c-*myc*. Elevated levels of amplification of c-*myc* maintained the tumorigenic phenotype in SW 613-S cells established from a human breast carcinoma (Lavialle et al 1988). Myc contributes to oncogenicity by binding to and inactivating growth arrest and down regulation of DNA repair inducible (GADD45a and GADD153) genes transcription (Barsyte-Lovejoy et al

2004). Deregulation of the c-myc expression has been demonstrated to inhibit differentiation of murine erythroleukemia cells (Bar-Ner et al 1992).

The *ras* family of oncogenes has three primary members (K-*ras*, H-*ras* and N-*ras*). They were first discovered as proteins encoded by retroviral oncogenes that had been picked up from the host genome by the Kirsten and Harvey rat sarcoma viruses. In normal cells, the activity of ras proteins is controlled by the ratio of bound GTP to GDP; GTP-bound ras is active and becomes able to engage downstream targets (Campbell et al 1998). In cancer, the majority of *ras* mutations involve codon 12 of the gene, with a smaller number involving other regions such as codons 13 or 61. Substitution of a single base-pair converts glycine into valine at amino acid number 12 of the Ras protein (Oudejans et al 1991). The mutational damage compromises the GTPase activity of ras and prevents GAPs from promoting hydrolysis of GTP on Ras; this leads to accumulation of an active form of GTP-bound Ras which acts as a molecular switch connecting extracellular signals with nuclear transcription factors (Valencia et al 1991). It has been found that Ha-ras 1 gene is activated in alimentary canal carcinomas of cattle (Campo et al 1990; McCaffery et al 1989). In human colon cancer cells, the expression of p21-ras has been found to be reduced by the inhibitory action of quercetin (Ranelletti et al 2000)

1.1.1.2 Tumour suppressor genes

The second class of genes are functionally antagonistic to oncogenes, and so they are called tumour suppressor genes. They are important normal constituents of the genome that regulate normal proliferation and growth. Tumour suppressor genes,

unlike oncogenes, are defined by their inactivation in the malignant phenotype and both alleles of the gene must be disrupted or deleted in order to remove normal constraints on cell proliferation. Two of the best studied tumour suppressor genes are the *Rb* gene, involved in human retinoblastoma, and the *p53* gene, which is universal in its distribution and activity.

1.1.1.2.1 Retinoblastoma

The existence of tumour suppressor genes was first suggested by cell hybridization experiments through the observation of a benign hybrid that resulted from fusion of normal cells with malignant cells (Sasabe and Inana 1991). Further support for the existence of tumour suppressor genes were obtained from cytogenic studies of families at risk for retinoblastoma. The study demonstrated that a chromosome deletion leads to loss of genes important in preventing tumour development (Brown 1997).

The retinoblastoma is a typical tumour suppressor gene and the major negative regulator of the cell cycle progression through its controlling of the expression of cyclin E. Cyclin E promotes transit from G1 to S phase (Geng et al 2001). It was initially identified by its association with the development of a malignant tumour which arises in the eyes of children. This ocular cancer is hereditary and results from losing both copies of *Rb* gene in embryonic retinoblasts (Friend et al 1986). The *Rb* gene comprises 180 kb of DNA on chromosome 13q 14, and encodes a 105 kD nuclear phosphoprotein Rb. The unphosphorylated form of Rb protein inhibits cell growth and represses genes required for DNA synthesis through its interaction with the E2F family of transcription factors (Nevins 2001). In addition, Rb/E2F interaction prevents formation of the cylin E/cdk2 complex that is required for S

phase entry and stimulation of replication machinery (Bartek et al 1997). The activity of Rb is regulated by cyclin D/cdk4, 6 at late G1, and phosphorylation of Rb leads to release of the E2F transcription factors. In DNA tumour viruses, human adenovirus E1A, SV40 T antigene and human papillomavirus E7 can form complexes with the unphosphorylated form of Rb of the host cell. Formation of the complex with viral protein leads to releasing of E2F from Rb/E2F complex, and free E2F promotes DNA synthesis in growth-arrested cells (Johnson et al 1993). In addition to its function as negative regulator of cell cycle, Rb can also contribute to cell death programme stimuli by its degredation by proteases and release of E2F which activates P53 (Tan and Wang 1998).

1.1.1.2.2 P53

P53 gene is a 20kb fragment of chromosome 17 p13.1 which encodes a nuclear phosphoprotein that has been associated with a variety of human cancers in its mutated forms. It was discovered through the ability of its encoded protein to complex with SV40 large T antigen in virus -transformed cells. P53 is normally in inactive form due to the nature of C-terminus and inhibition of its DNA binding domain. The activity of p53 is required during the cell cycle at G1 to maintain the replication checkpoint, and in the case of DNA damage, p53 arrests the cell cycle to allow repair of the damage by stimulating growth arrest. One of the p53-inducible genes, p53R2, encodes a ribonucleotide reductase that is directly involved in the p53 checkpoint for repair of damaged DNA (Tanaka et al 2000). If repairs fail, p53 intiates cell death programme (apoptosis) by stimulating pro-apoptotic gene such as (BAX) expression and inhibiting antiapoptotic gene (bcl-2); also p53 may induce apoptosis directly by changing mitochondrial membrane

potential through reactive oxygen (Li et al 1999). The activity of p53 is controlled by its negative regulator MDM2, which binds to p53 and enhances its degradation by ubiquitin ligase (Brady et al 2005).

Loss of p53 functions, whether by mutation or by degradation by DNA tumour virus oncoproteins, contributes to tumorigenesis. Uncontrolled cell cycle and failure of apoptosis are required for induction of primary tumour. The vascular endothelial growth factor (VEGF) is upregulated in the presence of mutated p53 (Kieser et al 1994a), and is downregulated in cells expressing wild-type p53 (Bouvet et al 1998). Upregulation of the positive regulator of angiogenesis (VEGF) (Kieser et al 1994b) allows metastasis by stimulation of secretion and activation of enzymes involved in degradation of the extracellular matrix (Lamoreaux et al 1998; Unemori et al 1992).

1.1.2 Virus and Cancer

Viruses have long been recognized as agents of neoplasia. In the first decade of the twentieth century, two oncogenic viral infections, avian leukosis virus and Rous sarcoma virus were identified in poultry (Rous 1979a). Later more viruses were identified as causing cancer. Viruses have been considered as contributing factors in at least 15% of human cancer worldwide. As tumour progression is a multistep process, viruses may be an essential step but alone they might not cause cancer. Certain co-factors are implicated in PVs associated tumor progression such as bracken fern, cigarette smoking and sexually transmitted diseases. Cigarette smoking, alcohol drinking and aflatoxins are risk factors for hepatocellular carcinoma. There are two classes of tumour viruses: generally the DNA tumour viruses inhibit the tumour suppressor genes whereas the RNA tumour viruses

contribute to cancer by activating oncogenes. All RNA tumour viruses belong to the retrovirus family and are classified in Class I and Class II (Coffin 1992). Class I RNA tumour viruses carry cellular oncogenes that were picked up by accidental recombination between viral and cellular DNA. Class II RNA viruses do not carry cell-derived oncogenes but they act by insertion of proviral DNA into the host cell genome close to cellular oncogenes. Unlike DNA tumour viruses, RNA tumour viruses can induce cancer within days after infection. This may be attributed to alteration of host genes during transduction and elevated level of their transcription as a result of association with powerful transcriptional promoter sequences of retrovirus LTRs, and this leads to unrestricted cellular proliferation (Ruddell 1995).

DNA tumour viruses are members of five distinct families that are capable of directly causing cancer in either experimental animals or humans. DNA viruses induce cellular proliferation, interfere with cellular differentiation and block apoptosis in postmitotic cells in order to meet their requirements for cellular enzymes for their genomes replication. Occasionally these processes lead to permanent cell transformation. The mechanism by which these viruses can induce unrestrained cellular proliferation is protein-protein interaction. This interaction is mediated by binding of viral oncoproteins to specific cellular proteins such as Rb and P53 proteins. In addition, DNA tumour viruses can also activate some cellular proto-oncogenes such as ras gene to accelerate cellular proliferation in order to meet their requirements for enzymes needed for replication. DNA viruses that cause cancer include hepatitis B viruses, SV40 and Polyomavirus, Adenoviruses, Kaposi's sarcoma-associated herpesvirus, Epstein-Barr virus, and papillomaviruses.

1.1.2.1 Human T-cell Leukemia virus Type 1 (HTLV-1)

After its discoveries in the US in 1980, the oncovirus HTLV-1 was aetiologically associated with both adult T cell leukamia (ATL) and a degenerative neurologic disorder known as tropical spastic paraparesis or human T-cell lymphotropic virus type I-associated myelopathy (HAM/TSP). It is currently estimated that HTLV-1 affects about 20 million people in the world and the majority of infected individuals remain healthy lifelong asymptomatic carriers. Approximately 5-10% HTLV-1 infected individuals develop either ATL or HTLV-1-associated myelopathy (HAM/TSP) an inflammatory disease of the central nervous system caused by increased viral replication which activates immune cells that subsequently enter the nervous system and cause injury by immunopathological mechanisms (Maroushek et al 1995). ATL is an aggressive malignancy of CD4 + T lymphocytes which presents 20-25 years after infection and develops in fewer than 5% of infected individuals (Murphy et al 1989). HTLV-1 displays an *in vivo* cellular tropism for various cell populations within the peripheral blood, suggesting that its receptor is ubiquitously expressed and its presence in these cells is thought to be a risk for ATL (Goon et al 2004). The pathogenesis of ATL has been linked to the effects of the viral protein Tax (Gatza et al 2005; Marriott and Semmes 2005). Tax is a 40-kDa phosphoprotein essential for both viral replication and cellular transformation. Tax protein can set infected T-cells into a continuous uncontrolled replication increasing the number of HTLV-I-infected cells by promoting proliferation and inhibiting apoptosis.

1.1.2.2 Kaposi's sarcoma-associated herpesvirus (KSHV)

KSHV was first discovered in 1994 as a member of the gammaherpesviruses subfamily (*Rhadinovirus* genus) based on similarities at the levels of nucleotide sequence, gene content, and genomic structure with the *Rhadinovirus* prototype, *Herpesvirus saimiri* (HVS) of the South American squirrel monkey (Moore et al 1996). They are double-stranded DNA viruses approximately 165kbp in size. Like other gammaherpesviruses, KSHV has numerous genes with homology to cellular host genes which have been captured during virus evolution. KSHV is associated with Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. The lesions that characterize Kaposi's sarcoma are not a result of neoplastic transformation of cells but arise from an excessive proliferation of spindle cells mixed with endothelial cells, fibroblasts, and inflammatory cells, accompanied by new blood vessel formation (Safai et al 1985). Lesions expand throught the dermis and these spindle cells form vascular channels which contain red blood cells and finally form an aggressive nodular growth with cutaneous and visceral spread. KSHV infection is characterized by a long latency period in B-cells and endothelial cell. While the vast majority of tumor cells from these malignancies contain virus in latent form, only a small subset of viral genes are actually expressed. One of these genes, the latency-associated nuclear antigen LANA-1, is a multifunctional protein encoded by the viral gene orf 73. LANA-1 is essential for the replication and maintenance of latent episomes; it acts as molecular bridge between the viral episome and cellular chromosome thereby ensuring equal segregation of virus into daughter cells (Ballestas et al 1999; Cotter II 1999). LANA-1 binds to p53, repressing its transcriptional activity and its ability to induce apoptosis (Friborg et al 1999). It is also able to abrogate the induction of

cell cycle arrest through its association with the phosphorylated form of pRb (Radkov et al 2000).

1.1.2.3 Epstein-Barr virus (EBV)

EBV is a member of the gammaherpesvirus family, lymphocryptovirus genera and is present in more than 90% of the human population. It preferentially infects B-lymphocytes and epithelial cells (Cohen 2000). Primary EBV infection is usually asymptomatic but can cause infectious mononucleosis. EBV is strongly associated with the development of several cancers, in particular with Burkitt's lymphoma (BL), Hodgkin's disease, and lymphoproliferative disorders which complicate immune suppression conditions. Latent EBV infection is an early event in the development of nasopharyngeal carcinoma (NPC) (Pathmanathan et al 1995). NPC is a tumour of poorly differentiated epithelial cells, highly prevalent in southern China, South-East Asia and North Africa. EBV was first identified by Epstein, Achong and Barr in 1964 in Burkitt lymphoma biopsies and its association with BL was demonstrated by the observation that sera from Burkitt lymphoma patients had higher titres to EBV antigens than controls. EBV genome is composed of linear double-stranded DNA, approximately 172 kbp in length. Only nine of the 80 genes encoded by EBV are expressed during latency. Among these genes, EBNA1 and LMP1 have been shown to be essential for infection and immortalization. EBNA1 is a DNA binding nuclear phosphoprotein, which is required for the replication and maintenance of the episomal EBV genome. EBNA1 protein contributes to latency via its glycine-alanine (Gly-Ala) repeat domain which acts as an inhibitor of MHC class I presentation by inhibiting antigen processing via the ubiquitin/proteosome pathway (Levitskaya et al 1995). LMP1 is the major

transforming protein of EBV, induces upregulation of antiapoptotic proteins (Henderson et al 1991) and has been shown to be essential for EBV-induced B-cell transformation *in vitro* (Wang et al 1985). LMP1 is prevalent in NPC in high risk areas, which would allow the virus to resist immune recognition and may in part contribute to prevalence of NPC in these populations. EBV associates with gastric carcinoma in approximately 10% of cases throughout the world (Takada 2000). Analysis of carcinoma biopsies has indicated that 100% of carcinoma cells in each case are EBV-infected and derived from proliferation of a single EBV infected cell suggesting that EBV plays an important role in the development of cancer.

1.1.2.4 Hepatitis

The hepatitis B virus (HBV), discovered in 1966, infects more than 350 million people worldwide. HBV belongs to the family hepadnaviruses and the HBV genome is a relaxed circular, partially double stranded DNA of approximately 3.2 kbp. Hepatitis B is a leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) accounting for 1 million deaths annually. HCC is the fifth most common cancer worldwide and the third most common cause of cancer death. Chronic infection with hepatitis B virus (HBV) is one of the major risk factors for liver cancer. Most HCCs (80%) arise in a cirrhotic liver and rates of progression to cirrhosis and hepatocellular carcinoma vary according to the state of the immune system, the age of the patient, the serologic stage of infection, and geographic and genetic factors (Brechot 2004). Hepatitis B is spread predominantly parenterally, through intimate personal contact, and perinatally. At greater risk of infection are intravenous drug users and children of mothers with HBV. Approximately 5% of the world's populations are carriers of HBV. Most

tumours arise in persons over 50 years of age, suggesting carcinogenesis is a protracted and (possibly) multi-step process and the integration of HBV-DNA into the hepatic genome is a significant event in hepatocarcinogenesis (Brechot 2004; Shafritz and Kew 1981). Aflatoxin B1 uptake, chronic alcohol consumption and cirrhosis are cofactors that increase the development of HCC in patients with chronic viral infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) (Dominguez-Malagon and Gaytan-Graham 2001).

1.2 Papillomavirus

Papillomaviruses contain a small molecular weight DNA genome, which is circular and approximately 8 kbp in length. The genome is contained in an icosahedral capsid, creating an extremely stable structure that is very resistant to environmental factors and thereby enabling effective transmission. In virions, the PV DNA is found associated with cellular histones to form chromatin-like complexes. The genome of PV is divided into three regions: a long control region and regions containing open reading frames corresponding to early and late genes. The viral genomes carry on average eight major open reading frames (ORFs), and these are expressed from a single strand of the double-stranded genome. Infection by papillomavirus is thought to occur through microwounds of the epithelium that expose cells in the basal layer to viral entry. The viral genome is normally maintained stably as autonomously replicating plasmids present at a low copy number, where it replicates in synchrony with the cell cycle (Gilbert and Cohen 1987). The replication of the viral genome and production of new infectious viruses is tightly aligned to the stratification and differentiation state of the epithelial cells. The viruses infect many species, including humans, rabbits, cows,

and dogs (Campo 2002). PV infection normally results in hyperproliferative lesions or benign tumors of epithelial cells of the skin or mucosa (warts) that usually persist for several months to one year and regress by activation of host immune system against viral antigen. However, occasionally, some lesions can persist and transform to carcinoma in the presence of genetic or environmental contributory factors. PV-associated neoplasias comprise anogenital carcinoma and skin squamous cell carcinoma in humans, skin cancer in rabbits and upper gastrointestinal cancer and urinary bladder cancer in cattle.

1.2.1 Papillomavirus life cycle

The life cycle of papillomavirus is intimately tied to the differentiation status of the host epithelium and has two distinct stages: the non-proliferative and proliferative stages. In the non-proliferative stage of the life cycle, initial infection requires access of infectious particles to the basal layer via the epithelium. This is thought to require a break in the stratified epithelium. Following infection, the virus maintains its genome as low copy number episome in the basal cells of the epithelium (Egawa 2003; Schmitt et al 1996). The pattern of viral gene expression in these cells is not well defined, but it is generally thought that the viral replication E1 and E2 proteins are expressed in order to maintain the viral DNA as an episome (Wilson et al 2002) and to facilitate the correct segregation of genomes during cell division (You et al 2004). Studies been carried out on HPV31 showed that failure to express full length E1 protein prevents episomal maintenance, and in cultured epithelial cells leads to the integration of viral genome into the host cell chromosome (Frattini et al 1996). It has been suggested that the viral genome is maintained extrachromosomally in the basal cells at around 50-100 copies per cell

and the viral early proteins (E1, E2, E6 and E7) are expressed at low level (De Geest et al 1993). In uninfected epithelium, basal cells exit the cell cycle after migrating into the suprabasal cell layers and undergo a process of terminal differentiation. Changes include the physical cross-linking of keratin intermediate filaments, the formation of cornified envelopes and secretion of lipids, which together allow the epithelial surface to form a physical barrier against the environment (Madison 2003). In virally-infected epithelium, as the host cells differentiate, the proliferative stage of viral life cycle occurs in the suprabasal layer of the epithelium. Amplification of viral genome requires expression of all viral early gene products. The E6 and E7 expression contributes to viral DNA amplification via their action to delay the terminal differentiation of the cells. E4 expression in this stage is thought to interfere with keratin network in order to facilitate virus particles assembly (Peh et al 2004). The expression of E5 in the proliferative stage contributes to viral DNA amplification by increasing the percentage of the infected suprabasal cells undergoing DNA synthesis (Genther et al 2003), it also activates late viral functions upon epithelial differentiation (Fehrmann et al 2003). Once viral genome amplification has been completed, transcription from the late promoter is activated. The virus encodes two structural proteins L1 and L2, which are organized into a 72 capsomere icosohedral shell at the sites of viral DNA replication, which is thought to facilitate packaging of the infectious virions (Becker et al 2004). Infectious virions are released into the environment as the upper layer of the epithelium is shed to infect new individuals or new sites in the same host.

Figure 1. The life cycle organization during productive infection by HPV types

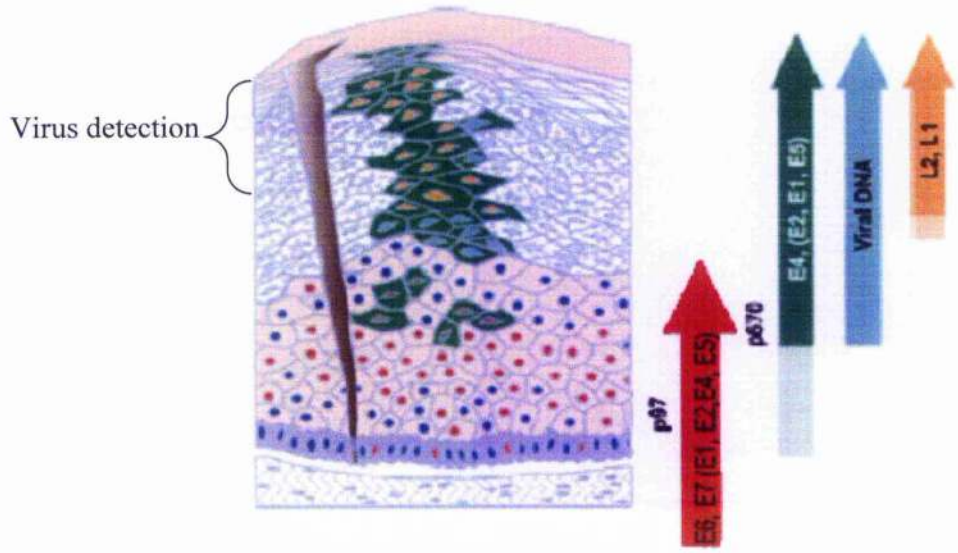


Figure 1. Life cycle organization during productive infection by HPV types.Diagram represents infected skin showing patterns of HPV-16 gene expression. Red arrow represents early genes expression (E1-E7) from the early promoter P97 at low levels in deep layers (basal and suprabasal) and elevated levels in intermediate layers (spinous). Green arrow represents an increase in abundance of early genes E1, E2, E4, and E5 expression in granular layers during viral DNA replication (Blue arrow). Yellow arrow represents expression of capsid proteins (L1, L2) from the late promoter P670. Viral DNA assembly in capsid and new infectious viral particles are released during shedding of dead cells. Picture from *J. Doorbar / Journal of clinical virology 32S (2005) S7-S15.*

1.2.2 Human papillomavirus

Human papillomaviruses (HPV) infection of epidermal or mucosal epithelial cells causes different types of warts such as laryngeal warts in young children (Cook et al 1973) and genital condylomas (Kumar et al 1990). Certain types of HPVs are detected frequently in anogenital cancers, particularly cancer of the cervix (Klaes et al 1999), head and neck squamous cell carcinomas (Ringstrom et al 2002). To date, over 100 different types of human papillomavirus have been identified, some of which are frequently associated with cancers and are considered high risk HPV (type 16, 18, 31 and 45), whereas infection by others causes warts and benign lesions and are considered low risk (types 6 and 11) (Munoz et al 2003). High risk HPVs are common in many epithelial derived cancers and are found in more than 99 % of all cervical carcinomas (Walboomers et al 1999). Infection by high-risk HPVs is not limited to the genital tract, since approximately 20% of oral cavity and oropharynx cancers contain HPV-16 DNA, the most common HPV type in genital cancers (Herrero et al 2003).

1.2.2.1 HPV-16

HPV-16 is clinically the most prevalent virus among the approximately 30 HPV types that infect the genital tract and is the virus most often associated with cervical cancer (Zur Hausen 1996). It is also associated with oral and laryngeal malignancies (Baez et al 2004). All HPVs have an overall similarity in the genomic organization which can be divided into two parts: the early genes and late genes regions. The early genes encode viral nonstructural proteins (E1, E2, E4, E5, E6, and E7 proteins) that are expressed early in infection in the basal and suprabasal layers. The late genes encode two capsid proteins (L1 and L2 proteins),

which are expressed later in the granular layer. The early and late regions are separated by a 850 bp long non-coding region rich in transcription factors sites called long control region (LCR) located between the L1 and E6 genes. The 3' end of LCR contains the origin of replication (ori) and binding sites for the transcription factor SP-1, YY1 and the 4 binding sites for viral E2. Gene expression of HPV-16 and other HPV types is epithelial specific and is controlled by cellular transcription factors binding to the LCR and by the virally encoded E2 (Steger and Corbach 1997); (Bouvard et al 1994b). Transcription initiates at several promoters, and the P_{97} promoter is the main early promoter (Tan et al 1994). Most of the early genes are transcribed from the P_{97} promoter, localized upstream of the coding region of the genome and regulated by transcription factors such as SP1 (Apt et al 1996). The late P_{670} promoter is differentiation-dependent and is only activated in the upper part of the epithelium, where transcription initiates in the middle of the coding region of E7 (Grassmann et al 1996), the late promoter encodes the capsid proteins (L1, L2) and E4.

Figure 2. Map of human papillomavirus type16 genome

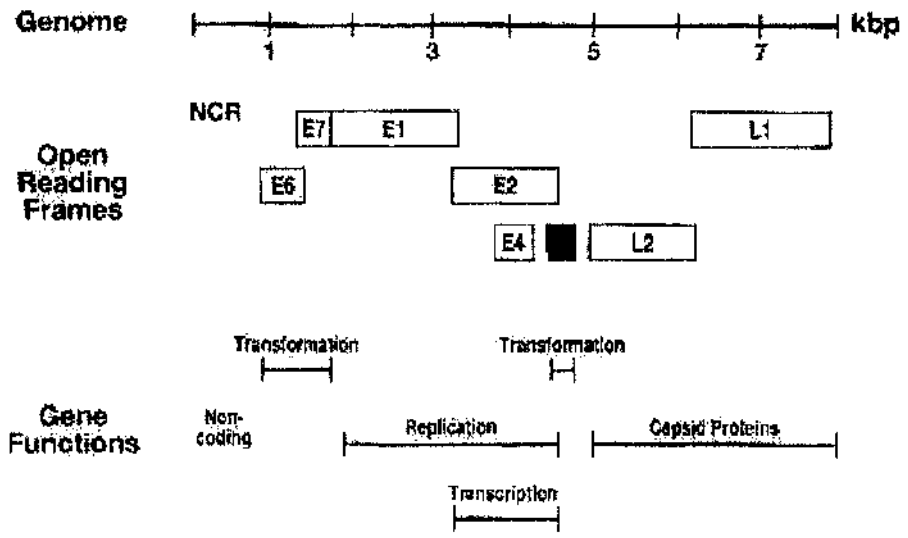


Figure 2. Map of human papillomavirus type 16 genome. The genome is marked in kilobase pairs (Kbp), and is shown linearized in the noncoding region (NCR). The NCR contains the origin of replication and enhancer and promoter sequences. All early (E1-7) and late (L1,L2) open reading frames are shown on the same strand of viral DNA. The highlighted box represents E5 ORF, the minor transforming gene in HPV while E6 and E7 are the major transforming genes. E1 and E2 are involved in viral gene transcription and replication. L1 and L2 express capsid proteins.

1.2.2.2 HPV and cervical cancer

The cervix is the narrow lower portion of the female uterus that extends into the vagina. Cancer of the cervix is a very common malignant disease in women worldwide. In many developing countries, and among female cancer deaths, cervical cancer is second only to the breast cancer. Although it is still a common disease, it has declined dramatically in incidence over the last several decades. For the most part, this is due to the widespread use of the Papanicolaou ("Pap") test. Various types of the human papillomavirus (HPV), a sexually transmitted infection, are responsible for the majority of cervical cancer cases and infection with high risk human papillomavirus (HPV) types, most frequently HPV 16, is the most significant risk factor in its aetiology (Bosch et al 1995).

Cervical intraepithelial neoplasia (CIN) is essentially a precursor to invasive cervical cancer, and is a condition characterized by new growth (dysplasia) in the normal epithelium of the cervix. The lesion arises from the transformation zone (T-zone) located between the mature epithelium of the exocervix and the columnar epithelium of the endocervical canal. Under physiological conditions, the columnar epithelium undergoes metaplasia to a stratified epithelium, during this process, it is vulnerable to external insults such as oncogenic HPV, resulting in the development of premalignant and possibly malignant cells rather than a normal epithelium (Arends et al 1998). The level of cellular changes is determined by histopathology and microscopy of Pap smears. CIN is recognised by disturbances of cellular maturation and stratification, and by the presence of cytological atypia. Infection of the genital tract by low-risk HPV (non-oncogenic) such as HPV-6 and 11 is associated with cervical condylomas, which can be distinguished from CIN by the absence of nuclear atypia in the basal layers of the epithelium. The high-risk HPV-

16 and 18 are found in 50-80 % of CIN II and CIN III lesions and up to 90% of invasive cervical cancer (Arends et al 1990). Infrequent detection of intermediate risk types such as HPV-31, 33 and 35 is associated with all grades of CIN and occasionally with cancer. CIN is classified histologically into three classes (mild (CIN I), moderate (CIN II) and severe intraepithelial neoplasia (CIN III), based on the proportion of the epithelium occupied by dysplastic cells. Initial infection with the high risk types (HPV-16 &18) causes low grade squamous intraepithelial lesions (CIN I), which is manifested by inhibition of the normal differentiation in the lower third of the epithelium, while differentiation occurs in the superficial two thirds of the epithelium. In CIN II, lesions move up to engage the middle third of the epithelium. The lesions may remain low grade or progress to severe dysplasia (CIN III), but the vast majority of CIN I and II lesions are cleared by the immune system. In CIN III, differentiation, which is minimal or even absent, occurs only in the most superficial third of the epithelium. CIN III therefore morphologically describes severe dysplasia and carcinoma *in situ*, which may persist or may start to invade below the basement membrane leading to invasive cancer. Although a certain correlation exists between the degree of severity of dysplasia and progression to a higher grade of CIN or cervical cancer, invasive cancer of the cervix can also develop from CIN I, while CIN II and CIN III cases do not always progress (Syrjanen 1996). Abnormalities usually attributed to the presence of HPV infection such as koilocytosis and epithelial multi-nucleation, are often present and are most conspicuous in CIN I and II, and minimal or absent in CIN III.

HPV and Vaccine development

Infection by certain high risk human papillomaviruses (HPVs) is the most significant risk factor in the development of malignant tumours of the anogenital tract. HPV infections are associated with the vast majority of cervical cancer cases of more than 95% of cases and the other 5% of cervical cancer cases may be unrelated to HPV infection (Zur 1991). The recognition that HPV infection plays the central etiologic role in cervical cancer has encouraged efforts to develop vaccines against HPV. HPV-16 is most commonly linked with cancer since it is present in 50% of cervical cancer and high-grade CINs and in association with HPV-18, 33, and 45 in 80% of cases worldwide. An effective HPV vaccine that targets HPV-16 and possibly other known oncogenic HPV types is therefore required to decrease the incidence of cervical cancer and its associated precursor lesions. Two main types of vaccine have been developed: prophylactic vaccines to prevent HPV infection and its cervical neoplasia, and therapeutic vaccines to induce viral clearance and regression of precancerous lesions. The clinical trials to develop prophylactic papillomavirus vaccine are composed of the viral L1 capsid protein which is expressed in eukaryotic or in prokaryotic systems and purified in the form of virus-like particles (VLPs) (Lowy and Schiller 1999). The L1 protein when is expressed in the absence of other papillomavirus genes can self-assemble into VLP that is morphologically and immunologically similar to infectious papillomavirus. Since the VLPs do not contain the papillomavirus DNA genome, they are not infectious and can not cause neoplastic changes in cells. VLP vaccines have been highly successful in the prevention and treatment of papillomavirus infection in animal models of cottontail rabbit virus, bovine virus and canine oral papillomavirus (Breitburd et al 1995; Kirnbauer et al 1996). Although,

prophylactic vaccine is mainly developed to generate neutralizing antibodies against HPV infection, it is also induces L1-specific T cell responses detectable by proliferation of both CD4+ and CD8+ T cells and in vitro production of both Th1- and Th2-type cytokines (Pinto et al 2003). Cell-mediated immune response participates in the generation and maintenance of protective B cell response and therefore may have an important role in the achievement of high neutralizing antibody titres. Furthermore, cytotoxic T cell responses to L1 may also be important for maximization of prophylactic efficacy by eliminating the number of HPV-infected cells that have escaped antibody neutralization (Zinkernagel et al 1996). Therapeutic vaccines are also being developed to protect HPV-positive patients against tumour development. Most efforts have been directed toward use of the E6 and E7 proteins, or peptides derived from them largely because these are the viral proteins that are retained and expressed in cervical cancer (Ling et al 2000). Papillomavirus genes can be introduced into cells as naked DNA, which is a relatively inefficient process, or as part of a viral vector such as vaccinia virus which usually more efficient. Therapeutic vaccines can also be developed against E1 and E2, which are required for the viral DNA to be maintained as an extrachromosomal element that is unintegrated in the host DNA, represent potential interesting targets for benign lesions ((Brandsma et al 2004). It has been indicated that tumour formation was prevented in mice after vaccination with HPV-16 L1/E7 fusion protein (Tian et al 2006). This kind of vaccination induces L1-specific antibodies and also L1- and E7-specific CTL responses (Kuck et al 2006). Recently, several studies have tested a quadrivalent recombinant vaccine referred to as Gardasil that consists of recombinant viral-like particles (VLPs) of HPV 6, 11, 16, 18 mixed with an aluminum-containing adjuvant. Gardasil prevents

HPV infection but does not treat it, so it has been approved for use in girls and women 9 to 26 years of age. Although, the vaccine is highly immunogenic and induces elevated antibodies titres, using this vaccine is a controversial and making concern for parents that the sexual activity of their children might be promoted if they got vaccinated. There is another HPV vaccine, Cervarix, which is currently under development. These vaccines constitute the most significant development in cervical cancer prevention so far, having the potential to reduce the incidence of cervical cancer by up to 70% (Widdice and Kahn 2006).

1.2.3 Bovine papillomavirus

Bovine papillomaviruses (BPV) predominantly infect cutaneous epithelium although some show specificity for the mucosal epithelium (e.g.BPV-4). The six well-characterised BPVs were originally classified into two groups based on their genome size, structure, tissue tropism, and the histology of the lesions: subgroup A and subgroup B. Subgroup A is comprised of BPV-1, BPV-2 and BPV-5. The feature that characterses these BPVs among all papillomaviruses is the ability to infect both epithelium and underlying fibroblasts, giving rise to fibropapillomas and may also induce papillomas in the less keratinized epithelium of the rumen. Subgroup B comprises BPV-3, BPV-4 and BPV-6, which infect only the epithelium and induce true papillomas. Papillomaviruses have recently been reclassified following the Greek letter nomenclature used for other virus families (de Villiers et al 2004). The epitheliotropic BPVs types 3, 4 and 6 are defined as Xi-papillomaviruses and BPV-1 and BPV-2 as Delta-papillomaviruses. It has been found that the genome of BPV-5 share homology with Xi-PVs in E1 ORF sequences and closely related to Delta-PVs in the genome size and structure (Bloch

et al 1994). These observations have led to the reclassification of BPV-5 in an intermediary subgroup of Epsilon-PV genus (de Villiers et al 2004). Like other PVs, BPV virions have constant structure and morphology of non-enveloped icosahadral structure, 55-60 nm in diameter. It contains the double stranded closed circular DNA complexed with cellular histones. The virion is composed of the major L1 and L2 capsid proteins. The BPV virion is characterised by exposing the C-terminus of L1 on the surface of virion, which probably has a role in infection and immunogenicity (Modis et al 2002). The genome differs in size and structure among BPVs; Delta-PV genome is 8 kbp whereas Xi-PV genome is approximately 7.3 kbp. The genome is divided into three regions: a long control region, the region containing early genes and region containing the late genes. The genome of Delta-PV is similar to most PVs, whereas Xi-PV lack the E6 gene which has been replaced by the E5 gene (Jackson et al 1991). In addition to the differences between Delta and Xi-PV, the LCR of Delta-PV contains 12 E2 binding sites (BS), whereas Xi-PV LCR contains only 4 E2 binding sites and shows similar arrangement patterns to the LCR of Alpha-PV (genital HPVs). Delta-PVs encode three oncoproteins, E5, E6 and E7, whereas Xi-PVs encode E5 and E7. E5 is the major transforming protein of BPV-1, followed by E6 with a modest role played by E7. In BPV-4, E5 and E7 are the transforming proteins and unlike BPV-1 E7 whose expression is restricted to the cytoplasm and nucleus of the basal and suprabasal layers, BPV-4 E7 is major transforming protein expressed in all layers and stages of papillomas. Both BPV type 1 and 2 predominantly induce fibropapillomas on the skin of cattle and experimentaly are capable of transforming primary bovine fibroblast or epithelial cells to complete oncogenicity, both as virus and as DNA (Campo 1992), but BPV type 4 DNA alone is not sufficient to induce

transformation of primary bovine cells and needs the cooperation of an activated *ras* oncogene. BPV infection is readily transmitted in herd animals through direct contact of abraded skin. Natural infection of horses with BPV-1 often occurs after placing the horses in stalls previously housing infected cattle. Sexual transmission of venereal warts in cattle apparently occurs since these warts are rare when using artificial insemination. PVs virions have also been detected in milk suggesting that mammary epithelium may also be susceptible to infection and may represent a potential route for vertical infection.

Table 1: Bovine Papillomaviruses (BPVs) and the tumours they cause

Genus	type	Tumour caused
Delta (δ)-PV	BPV-1	Fibropapillomas of paragenital areas
	BPV-2	Fibropapillomas of skin; Fibropapillomas of alimentary canal
Epsilon (ε)-PV	BPV-5	Fibropapillomas of teats and udders
Xi-PV (ξ)	BPV-3	Papillomas of skin
	BPV-4	Papillomas of alimentary canal
	BPV-6	Papillomas of teats and udders

From de Villiers et al., 2004

Although, the majority of cases (warts) caused by different types of BPVs are usually eradicated by a cell-mediated immune response directed against viral antigens, few cases have been associated with progression to squamous cell carcinoma. BPV-1 and BPV-2 which cause skin warts, also cause cancer of the urinary bladder, BPV-4 has been found as the causative factor of upper alimentary

canal cancer (Campo 1997), BPV-1 also induces penile papillomas that can progress to cancer (Jarrett 1985). As cancer is a multi-factorial disease and several steps are required before fully transformation is achieved, both urinary bladder cancer and upper alimentary canal cancer develop as a result of the interaction between the virus, environmental factors (bracken fern) and host cell factors activation. BPV-3, BPV-5 and BPV-6 can cause significant economic consequences particularly infection by BPV-6, where papillomas spread around the primary tumour to form large infected area, which interfere with milking and suckling. These lesions can get ulcerated, become infected with bacteria with susceptibility to cause mastitis.

1.2.3.1 BPV-4 genome

BPV type 4 is a tissue-specific virus that infects only the mucosa of upper alimentary tract. All sites from the tongue to the rumen can be affected (Campo et al 1980). BPV-4-associated papillomas are widespread in nature, they have been observed in different geographical areas. These benign tumours can become a focus for transformation to squamous cell carcinomas in animals feeding on bracken fern (Campo et al 1994). The contribution of viral, immunological and chemical factors in neoplastic progression of BPV-4 papillomas was first established in the field, and the high cancer incidence overlaps with grazing areas in which there is severe infestation of bracken fern (Jarrett et al 1978). The specificity of the virus for mucosal epithelium could be due to cellular receptors used by the virus to enter the cells or possibly to cellular factors restricted to the alimentary tract that are needed for viral replication (Jarrett 1985). The BPV-4 genome consists of a single molecule of double-stranded, circular DNA containing

approximately 7261 base pairs. The entire BPV-4 genome is required for the induction of papillomas (Moar et al 1986), but the presence of the BPV-4 DNA is not necessary for the progression to, or the maintenance of, the transformed state (Campo et al 1985). The BPV-4 genome is functionally divided into three regions, at the end of each coding region there are polyadenylation sites: the regions encoding the early and late gene products are separated by a noncoding region of 500 to 1,000 bp (about 12% of the viral genome), called the long control region (LCR). The LCR regulates transcription and replication of the viral DNA and contains a number of binding sites for transcription factors including virus-encoded E2. The BPV-4 long control region (LCR), and those of human papillomaviruses (Desaintes and Demeret 1996), have similar organizations: an enhancer region, a promoter region, and a highly conserved distribution of E2 DNA binding sites (BS). The BPV-4 enhancer shares similarity both in size and position to that of HPV-16 and HPV-18 (Morgan et al 1999). The enhancer of these papillomaviruses is epithelial cell specific, as it fails to activate transcription from hetrologous promoter in non epithelial cell types. The promoter region of BPV-4 is 127 bp and contains the origin of replication, three binding sites for viral E2, a TATA box and an initiator element. BPV-4 promoter region does not contain binding sites for the cellular factors SP1 (a positive regulator), YY1 (negative regulator) and CDP/Cut, the repressor of HPV-16 transcription and replication. It has been found that the family of C/EBP transcription factors are implicated as both positive and negative regulators of transcription from HPV and BPV-4 LCR (McCaffery and Jackson 1994). It has also been shown that BPV-4 LCR contains a 21bp cis element designated QRE-1, which is located immediately downstream of the TATA box. QRE-1 up-regulates transcription from LCR and thus an increased expression of

viral oncoprotein in response to quercetin exposure (Connolly et al 1998). In the LCR of mucosal PV, including human papillomavirus type 16 (HPV-16), there are four E2 binding sites (BS1–4). (Desaintes and Demeret 1996). Binding sites (BS1, 2) are located immediately upstream from the TATA box and separated from each other and from the TATA box by 3 or 4 base pairs (bp). Binding site (BS3) is beside the E1 DNA-binding site which involved in the regulation of viral DNA replication, and (BS4) is a further 300–400 bp upstream. Mutational analysis of an individual E2 shows that E2 binding to sites 1 and 2 results in transcriptional repression, while E2 binding to sites 3 and 4 leads to transcriptional activation (Demeret et al 1997).

Figure 3. Map of bovine papillomavirus type 4 genome

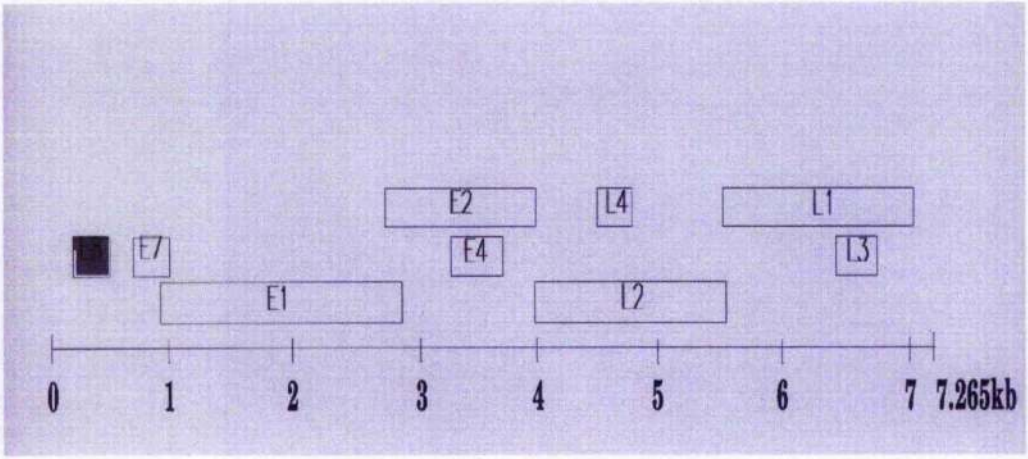


Figure 3. Map of bovine papillomavirus type 4 genome. The genome is marked in kilobase pairs (Kbp). The boxes represent open reading frames (ORFs) which encode for early genes (E1-7) and late genes (L1, L2). All the ORFs are on the same strand of viral DNA. The highlighted box represents E5 ORF that occupies the position of the missing E6 ORF in Xi PV *genus*. E5 ORF was orginally named E8 and because of similarities with BPV-1E5, it was renamed E5.

1.2.3.2 BPV-4 and squamous cell carcinoma of upper gastrointestinal tract (GIT)

Papillomaviruses are necessary, but often not sufficient factors in the induction of squamous cell carcinomas of cutaneous and mucosal epithelia (Jackson et al 1993). BPV-4 infects the mucous epithelium of the upper alimentary tract of cattle and induces benign proliferative lesions (papillomas). In healthy cattle, the papillomas develop and persist for approximately 1 year and are then rejected by a cell mediated immune response (Knowles et al 1996). However, in cattle grazing on

braken fern the papillomas can transform and progress to squamous cell cancer (Campo and Jarrett 1986). Braken fern contains immunosuppressants and mutagens. Bracken-eating cattle become chronically immunosuppresed and incapable of mounting an appropriate immune response against the virus or virus infected cells (Campo 1997). An incidence of upper and lower GI tract cancers has been found to occur at high frequency in very restricted geographical areas such as Nasampolai valley of Kenya and the western highlands of Scotland where cattle graze on bracken-infested land (Jarrett et al 1978; Plowright et al 1971). The bracken mutagens are probably implicated for the observed activated ras in alimentary and urinary bladder carcinomas (Campo et al 1990), mutation of p53 (Scobie 1996) and increase in epidermal growth factor receptors. Quercetin is a powerful flavonoid mutagen widespread in nature, is found in high levels in bracken fern (Bjeldanes and Chang 1977; Nakayasu et al 1986). It binds DNA causing single DNA breaks (Fazal et al 1990), DNA rearrangements (Suzuki et al 1991) and chromosomal damage. Moreover, quercetin arrests normal proliferating cells in the G1 phase of the cell cycle (Connolly et al 1998). It has been shown that exposure to a single dose of quercetin can cause anchorage-independent growth of partially transformed PalF cells by E7, achieve oncogenic transformation and the cells can induce tumours in nude mice (Cairney and Campo 1995; Pennie and Campo 1992). Therefore, quercetin substitutes for E5 in inducing independence from substarte (see 1.2.6), for E6 in conferring immortality and for mutant p53 in inducing oncogenicity (see 1.2.5.3.1). The synergy between quercetin and BPV-4 E7 has been supported by induction of trans-activation of the transcriptional promoter/enhancer (LCR) via QRE-1 bound by quercetin (Connolly et al 1998). Up-regulation of the LCR increases the expression of viral transforming proteins,

most likely E7. In the normal cells, exposure to quercetin induces cell cycle arrest in G1 but in keratinocytes transformed by HPV-18 E6 and E7, which is a similar situation to that of PalF cells transformed by BPV-4 E7, quercetin upregulates LCR and increases E7 expression. E7 inhibits the cell cycle regulator $p27^{kip1}$, preventing the quercetin-induced cell cycle arrest in G1, propelling the cell through the cell cycle (Beniston and Campo 2005).

1.2.4 Papillomavirus viral proteins

1.2.4.1 Viral DNA replication proteins

Viral DNA replication occurs in the infected cell using the cellular DNA replication machinery. Viral infection of the basal cells leads to activation of viral gene expression which results in production of approximately 50 extrachromosomal copies of the viral DNA per cell. Among the first viral proteins to be expressed are the replication factors E1 and E2. These proteins form a complex that binds to sequences at the viral origin of replication and also acts to recruit cellular polymerase and accessory proteins to mediate replication (Conger et al 1999; Frattini and Laimins 1994; Mohr et al 1990).

1.2.4.1.1 E1 protein

The E1 ORF encodes a 68 kDa protein essential for viral DNA replication. The full-length E1 product is a phosphorylated nuclear protein that binds to the origin of replication positioned at the 3'terminus in the LCR of papillomaviruses. The E1 protein carries multiple activities required for the initiation of DNA replication. It contains blocks of homology in the C-terminal amino acids with the SV40 replication protein large T antigen and other polyomaviruses. E1 protein of

papillomavirus has ATPase dependent helicase activity that functions to allow the separation of viral DNA strands ahead of the replication complex (Hughes and Romanos 1993). By itself, E1 weakly binds the origin of replication, but complex formation with E2 facilitates loading E1 onto the origin. E1 proteins also bind DNA polymerase and help to recruit cellular replication complex to the viral origin of replication (Masterson et al 1998). E1 may also promote replication initiation and elongation by alteration of viral chromatin structure and disruption of nucleosomes at the replication fork (Swindle and Engler 1998). As E1 transcription results from activation of early promoter in the basal cells, it is not fully understood yet how the E1 activity is regulated in differentiating cells, but studies with HPV-11 have shown that expression of E1 transcripts shifts from the early to late promoters, resulting in increased E1 expression (Deng et al 2003).

1.2.4.1.2 E2 protein

The products of the papillomavirus E2 open reading frame play a key role in the regulation of the viral cycle. E2 proteins can activate or repress viral promoters by several distinct mechanisms. E2 proteins are approximately 50 KDa in size and function as dimers. The C terminus contains a DNA binding domain which interacts with E1 (Chen and Stenlund 2000). E2 dimers bind to consensus sequences (ACC6GGT) called E2 binding sites located in LCR. On infection, early gene transcription is activated primarily by cellular transcription factors binding these sequences in LCR. At low concentrations E2 activates early gene expression, while at high concentration, it functions directly as an active repressor and inhibits HPV transcription at srages after TATA box recognition by TBP or TFIID (Hou et al 2000). This regulation of viral expression by E2 contributes to control of copy

number in undifferentiated cells. When the late promoter is switched on at the differentiation stage, at which its activation is differentiation dependent and is not repressed by E2, this results in transcription shifting from the early to the late promoter leading to increased levels of E1 and E2 expression, and an increase in viral DNA amplification (Klumpp and Laimins 1999). E2 may also form complexes with C/EBP transcription factors which regulate many promoters of genes involved in differentiation (Hadaschik et al 2003). Apart from its role in the regulation of transcription, overexpression of E2 protein can induce apoptosis by a p53 independent mechanism (Dowhanick et al 1995). In addition, introduction of heterologous expression vectors for E2 in cervical carcinoma cell lines (HeLa cells) containing HPV-18 results in a suppression of endogenous E6 and E7 gene transcription, leading to senescence of these cells (Goodwin and DiMaio 2000; Hwang et al 1993). This indicates that the continued expression of E6 and E7 is required to maintain the transformed phenotype.

1.2.4.1.3 E4 protein

The E4 gene product is found primarily in the cytoplasm of keratinocytes, where it is extremely abundant, but certainly detected in the nucleus and at perinuclear sites in some infections. The E4 ORF is translated from spliced transcripts as a fusion with the first five amino acids of E1 to generate E1^E4 fusion protein. The E4 ORF lacks an initiator AUG codon and uses the E1 sequence for translation initiation (Howley 1996). All papillomaviruses express E1^E4 proteins in the late phase of viral life cycle. E1^E4 proteins from the high-risk types associate with keratin networks in the cells, suggesting that the five N-terminal E1 amino acids play a major role in the interaction of E4 proteins with intermediate filaments

(Rogel-Gaillard et al 1993). In transient-transfection assays, overexpression of E1^E4 proteins induce collapse of keratin (Doorbar et al 1991), but in natural infection only limited amount of collapse has been observed. It has been indicated that E4 proteins play a significant role in promoting viral genome amplification, maintenance of S phase during differentiation and expression of capsid proteins, these observations were correlated with initiation of E4 expression in suprabasal epithelial cells (Peh et al 2004; Wilson et al 2005).

1.2.4.2 Capsid proteins

Papilloma virions contain two virally encoded proteins, L1 and L2, which are synthesized late in the infectious cycle. These two proteins encapsidate a histone-associated closed circular, double-stranded DNA. The L1 ORF encodes the 56-60kD major capsid protein. The minor capsid protein L2 has a molecular weight of 76-78kD. The N-terminal domain of the L2 protein is highly conserved among different papillomaviruses. Since L1 protein forms into capsomeres in the cytoplasm, viral DNA packaging should occur in the nucleus. After translocation of L2 to the nucleus, L2 has been proposed to be responsible for recruiting L1 capsomeres into nucleus and also recruits the viral genome to assembly sites (Day et al 1998). The outer shell of the virion contains 72 pentamers of L1, centered on the vertices of a T = 7 icosahedral lattice (Baker et al 1991; Trus et al 1997). L2, a largely internal protein, is present at about 1/30 the abundance of L1(Kirnbauer et al 1993). The L1 major virion structural proteins have recently been shown to self-assemble into virus-like particles when expressed in insect cells. These particles are the basis for a prophylactic vaccine to prevent genital HPV infection (Lowy et al 1994).

1.2.4.3 Papillomavirus transforming proteins

1.2.4.3.1 The E6 protein

Analysis sequence of genus Xi (ξ) of bovine papillomaviruses including BPV-4 revealed that the virus does not possess an E6 ORF and failure to induce full transformation may be due to lack of this gene. The E6 functions might be performed by another viral or host protein. Furthermore, the position that might be expected to be occupied by E6, between the long control region and the E7 ORF, contains the E5 ORF (Jackson et al 1991). Apart from BPV-4 and other member of genus Xi, animal and human papillomaviruses have an E6 gene and its product is one of the major transforming proteins in HPV-16. The HPV-16 E6 protein is a 151 amino acid protein, containing two zinc finger domains. E6 induces several important changes in the host cell that affect both the normal viral life cycle and the process of immortalization. E6 can efficiently immortalize human mammary epithelial cells and cooperates with E7 to immortalize primary human foreskin keratinocytes (HFK) (Kiyono et al 1998). An important function of E6 in tumorigenesis is its binding to and inactivation of p53 (Werness et al 1990). p53 is a transcription factor that stimulates the expression of genes involved in cell cycle arrest and apoptosis, such as the cyclin dependent kinase inhibitor, p21 (Ko and Prives 1996). E6 induces p53 degradation via a process mediated by an ubiquitin ligase called the E6-associated protein (E6-AP) (Huibregtse et al 1991). E6 can also indirectly downregulate p53 activity through its association with p300/CBP, which is a transcriptional coactivator of p53 that gathers transcription factor and the basal transcription machinery at gene promoters (Zimmermann et al 1999). Another major function of the HPV-16 E6 is their ability to activate the expression of the catalytic subunit of the telomerase, hTERT (Klingelhutz et al 1996).

Telomerase is a four subunit enzyme that adds hexamer repeats to the telomeric ends of chromosomes, following cell division which leads to shortening of telomerase. This process is only restricted to embryonic cells and absent in somatic cells which undergo senescence after cell division (Liu 1999). Reactivation of hTERT expression occurs in most cancer and leads to reconstitution of telomerase activity. It has been shown that E6 binds to Myc and its cofactor Max, leading to transcriptional activation of the hTERT promoter (Veldman et al 2003). In the productive life cycle, the main role of E6 is to facilitate viral replication, as shown by the observation that E6 of HPV-31 and HPV-6 is required to maintain stably episomal replication (Thomas et al 1999) probably, by a disruption of the mechanisms that prevent the maintenance of extrachromosomal DNA.

1.2.4.3.2 The E7 protein

The E7 is a potent transforming protein which is well conserved among different PVs. In BPV-4, E7 has been detected in most papilloma cell layers, in the nucleus of the basal and suprabasal cells and in the cytoplasm of the spinous and squamous layers (Anderson et al 1997). In HPV, E7 proteins of both low- and high-risk are found predominantly in the nucleus. They are approximately 100 amino acids in size. The protein is divided into domains; CR1 at the N terminus; CR2, which contains an LXCXE motif that binds the Rb protein and CR3, which contains two zinc finger-like motifs. The CR1 and CR2 domains of E7 have sequence homology to adenoviral E1A CR1 and CR2 domains that also bind to Rb proteins (Phelps et al 1988). E7 proteins contribute to cell transformation through their binding to and abrogation of the function of members of the retinoblastoma family of proteins. The ability of E7 to inhibit the transcription repressive functions of the Rb proteins

leads to stimulation of cell cycle progression, even in the presence of cellular arrest signals (McCance 2005). In BPV-4, E7 ORF encodes a polypeptide homologous to HPV-16 E7 with recognizable p105Rb binding domain and two cys-x-x-cys zinc binding motif (Dyson et al 1989; Munger et al 1989). Recently, it has been demonstrated that Rb binding domain is shared by all E7 proteins with exception of E7 proteins of fibropapillomaviruses (Narechania et al 2004) and therefore the ability of BPV-4 E7 to transform cell may be due to its binding to Rb protein whereas, BPV-1 E7 which does not have Rb binding domain, is not able to transform cells unless it cooperates with E6. E7 has also been associated with histone deacetylases (HDAC), AP-1 transcription factors, cyclins, cyclin-dependent kinases (cdks), and cdk inhibitors. These associations contribute to the ability of E7 to induce cellular proliferation, immortalisation and transformation. Cyclin E-cdk2 is essential for initiation of DNA synthesis and can activate cell cycle progression in the absence of cyclin D-cdk4 underlining this complex as a potential target by which E7 propels cell cycle re-entry. The expression of E7 induces dysregulation of cyclin E expression at transcriptional and post transcriptional levels, suggesting that E7 may affect cyclin E-cdk2 activity via increased synthesis of cyclin E (Zerfass et al 1995). Additionally, E7 inhibits the cell cycle regulator $p27^{kip1}$, preventing cell cycle arrest in G1 in quercetin-treated cells (Beniston and Campo 2005). E7 can also interact with members of the Ap-1 family of transcription factors including c-Jun, JunB, JunD and c-Fos. Ap-1 transcription factors mediate early mitogenic effects and are implicated in keratinocyte and myeloid cell differentiation. Mutational analysis revealed that the E7 zinc finger motif is involved in c-Jun/ E7 interaction. It has been demonstrated

that the binding of E7 to c-Jun increases the ability of E7 to transform rat embryo fibroblasts (REFs) in the presence of activated ras (Antinore et al 1996).

1.2.5 Papillomavirus E5

The analysis of the DNA sequence of the papillomavirus genome revealed that the E5 ORF commonly is located at the 3' end of the early region before the L2 ORF and overlaps the E2 ORF in the early region. However, in the epitheliotropic Xi-papillomavirus BPVs the E5 ORF (originally designed E8) occupies the position of the missing E6 ORF at the 5' end of the early region, between the long control region and the E7 ORF (Jackson et al 1991). E5 genes of PVs encode small highly hydrophobic proteins ranging from 42 amino acids in BPV-4, 44 amino acids in BPV-1 to 83 amino acids in HPV-16. The gene is not well conserved at the DNA level between HPVs or BPVs, although there is conservation of the physio-chemical properties in that the proteins are all highly hydrophobic and membrane bound (Conrad et al 1993). The HPV-6 and HPV-11 E5 proteins share structural properties with the BPV-1 E5 protein: they contain a Cys-X-Cys sequence near the carboxy terminal and glutamine residue within the hydrophobic domain. Both these regions have been shown to be critical for the transforming activity of BPV-1 E5 (Horwitz et al 1989). The most studied PV E5 proteins in animal and human are BPV-1 and HPV-16 respectively. In BPV-1, 95 to 99 % of the *in vitro* transforming activity of BPV-1 DNA is due to the E5 gene and the remaining percentage of transforming activity is due to E6 (Neary and DiMaio 1989; Schlegel et al 1986). BPV-1 E5 can be divided into two domains: N-terminus and C-terminus. The N-terminus, which is a hydrophobic domain, includes 30 amino acids and is thought to exist in an α-helical conformation important for membrane

anchoring and cell transformation. The carboxy-terminal sequence is generally hydrophilic and contains two cysteine residues. The Cys-X-cys sequence is conserved in E5 protein of bovine and human papillomaviruses oligomeres via disulfide bond, contributing to the transforming activity of E5 (Schlegel et al 1986). The E5 protein is thought to be a type II transmembrane protein that is localized largely to the membranes of the endoplasmic reticulum and Golgi apparatus of transformed cells and exists as a dimer of two identical subunits linked by disulfide bonds involving cysteine residues in the carboxyl-terminal third of the protein which appears to extend into the lumen of these organelles (Burkhardt et al 1989; Horwitz et al 1988). In naturally infected bovine cells, E5 is expressed in early stages of infection, where it has been found that the majority of basal cells within a wart are infected with virus (Burnett et al 1992). However, large amounts of E5 has been detected in the differentiated keratinocytes and the role of E5 in this stage is thought to alter the normal physiology of the host differentiated keratinocytes to promote viral DNA replication and capsid protein expression. In HPV-16, E5 gene ecodes a hydrophobic transmembrane protein localized mainly in the endosomal membranes and Golgi apparatus. E5 proteins encoded by several HPVs which infect the genital region (e.g., types 6, 11, 16, 18, 33) exhibit a conserved hydrophobic structure, but not a conserved amino acid sequence (Bubb et al 1988). The analysis of amino acids sequence of HPV-16 E5 indicates that the protein is composed of three distinct hydrophobic domains that are capable of spanning a membrane three times. Unlike BPV-4 and BPV-1 E5, HPV-16 E5 protein has a lower oncogenecity compared with the two major transforming proteins E6 and E7. E5 can transform rodent fibroblasts, but is unable to immortalize keratinocytes, although it extends the *in vitro* life span of these cells

(Straight et al 1993). The HPV-16 E5 protein has been shown to cooperate with E7 protein to induce proliferation of 3T3 cells in the presence of certain growth factors for transformation and stimulation of DNA synthesis, suggesting that E5 may cooperate with the E7 gene to stimulate cell proliferation *in vivo* (Bouvard et al 1994a). Detection of E5 mRNA as well as an elevated levels of its gene product in low grade of CIN suggests that E5 has a significant role in the viral infection and early steps of cellular transformation (Kell et al 1994; Stoler et al 1992). In well-developed carcinomas, E5 has been found to be frequently deleted upon incorporation of viral DNA into the host genome, suggesting that E5 is not necessary to maintain the malignant state. It has been shown that expression of E5 gene is regulated by transcriptional factors encoded by the E2 ORF in C127 cell transformation assays. E2 transactivation results in an increase transcription from the P2443 promoter whose transcripts encode the E2 transactivator as well as the E5 oncoprotein, whereas the P2443 promoter activity could be inhibited by the E2 trans-repressor (Hermonat et al 1988). E5 gene is required for efficient focus formation and elimination of E2 transactivation activity, removal of the LCR, or increased of E2 repressor synthesis causes dramatic decreases in the expression of the E5 gene and severely inhibits focus formation (Prakash et al 1988). In BPV-4, E5 and E7 are the major transforming proteins. The E5 ORF is located at 5' end of the early genes and encodes the smallest PVs E5 proteins; it is only 42 amino acids long. BPV-4 E5 protein comprises two distinct domains: the N-terminus is a highly hydrophobic, 30 amino acids residues with the capacity to form an α-helix and a hydrophilic C-terminal tail of 12 amino acids residues (Jackson et al 1991). The BPV-4 E5 protein is localised in endoplasmic reticulum and Golgi apparatus and it is expressed in deep layers of early stages papillomas of the upper alimentary tract

(Anderson et al 1997). E5 proteins from both animal and human PVs possess biological activities with varying degree of efficiency in the transformation of mammalian cells. E5 contributes to cell transformation through interaction with several cellular proteins, including the epidermal growth factor receptor (EGF-R), the human receptor for colony stimulating factors (CSF-1) and the 16 kDa subunit of the vacuolar H^+-ATPase. In addition, BPV-1, BPV-4 and HPV-E5 interact with platelet-derived growth factor receptor PDGF-R.

1.2.5.1 BPV-1 E5 and platelet-derived growth factor receptor

The E5 protein does not have intrinsic enzymatic activity but rather induces transformation by modulating the activity of cellular membrane proteins that regulate cell growth. It has been suggested that E5 protein targets the growth factor receptors since co-transfection of E5 gene with human epidermal growth factor receptors genes in NIH3T3 cells resulted in transformation of cells correlated with delayed turnover of activated EGF receptor in co-transfected cells (Martin et al 1989). Moreover, expression of E5 induced DNA synthesis in quiescent fibroblasts after being treated with E5 and purified growth factors (Settleman et al 1989).

The platelet derived growth factor beta receptor (PDGF-β) activated by the BPV-1 E5 protein has elevated levels of tyrosine kinase activity with the receptor activated PDGF. In addition, PDGF receptors whether activated by E5 or by PDGF have been shown to be associated with various SH2 domain containing cellular proteins that play crucial roles in response to PDGF (Cohen et al 1993; Petti et al 1991). It has been shown that cells lacking endogenous PDGF β receptor are not susceptible to E5-induced transformation. However, co-expression of the E5 causes receptor activation and transformation and provides an anti-apoptotic signal

in the face of growth factor deprivation (Nilson and DiMaio 1993). It has been reported that certain mutants of E5 are able to transform cells without binding to or activating the PDGF β receptor, suggesting that these mutants utilize alternative PDGF β receptor-independent mechanisms to transform cells (Schapiro et al 2000; Suprynowicz et al 2000). However, recent data indicated that at least some of these mutants interact productively with the PDGF β receptor (DiMaio et al 2000). The E5 protein and the PDGF β receptor form a stable complex in cells. Unlike PDGF, which binds to the extracellualr domain of the receptor, E5 protein interacts with transmembrane and juxtamembrane domains of the PDGF β receptor (Cohen et al 1993; Staebler et al 1995). It has been proposed that formation of the complex E5/ PDGF β receptor requires an electrostatic bond between juxtamembrane lysine in the PDGF β receptor and aspartic acid 33 of the BPV E5 protein and hydrogen-bond between a transmembrane threonine in the receptor and glutamine 17 of the E5 protein (Horwitz et al 1988; Klein et al 1998). The requirement of the glutamine 17 and aspartic acid 33 in E5 and PDGF β receptor interaction may explain the inability of the E5 protein to activate the PDGF alpha receptor, which lacks the essential threonine and lysine (Staebler et al 1995) Complex formation also requires dimerization of E5 protein and glutamine 17 plays a role in stabilizing the E5 dimer (Klein et al 1998). It has been demonstrated that PDGF β receptor is activated, complexed and colocalized with E5 protein in neoplastic epithelial cells from bovine urinary bladder tumours (Borzacchiello et al 2006).

1.2.5.2 HPV-16 E5 and EGF-R

Viruses have evolved mechanisms to modulate cellular signaling pathways to reprogram host cells to support their life cycles or modulate host defense responses

(Burgert et al 2002). Alterations in the expression and/or activity of epidermal growth factor receptor (EGFR) family members are common in many types of cancers, and their activity contributes to tumorigenicity. Epidermal growth factor is the ligand for EGF receptor (EGFR), a transmembrane receptor protein present on all epithelial cells. The binding of EGF to the extracellular domain of the receptor intiates receptor dimerization and stimulates the activity of tyrosine kinase localized on the intracellular domain of the receptor resulting in autophosphorylation and initiation of downstream cascades leading to the stimulation of DNA synthesis (Chen et al 1987; Moolenaar et al 1988; Schlessinger et al 1988). PV E5 contributes to cell transformation via its ability to inhibit down-regulation of the epidermal growth factor (EGF) receptor (EGF-R). It has been reported that the cooperation between E5 and high levels of EGF receptors is associated with inhibition of receptor degradation and persistence of activated receptors on the cell surface (Martin et al 1989). Recent studies have demonstrated that HPV-16 E5 has activities to transform rodent fibroblasts and stimulates mitogenesis in human epithelial cells and these activities are enhanced by stimulation of EGF. Furthermore, these activites may be attributed to the ability of HPV-16 E5 in human keratinocytes to increase the number of EGFRs at the cell surface, inhibit degradation of the receptor in endosomal vesicles, increase receptor kinase activity and cause the recycling of the receptor to the cell surface (Straight et al 1993). It has been revealed that inhibition of the EGFR down-regulation by E5 results from the defective endosomal acidification in E5-expressing cells (Straight et al 1995). The lack of acidification may be due to interference with H^+-ATPase pump which may be associated with the ability of E5 to bind to a 16-kDa proteolipid, which is a component of the vacuolar proton-ATPase pump (Conrad et

al 1993). This binding has been suggested to cause delaying of EGFR degradation and an increased recycling of EGFR to the cell surface. It has been suggested that HPV E5 proteins contribute to proliferation of keratinocytes by enhancing the mitogenic activity of autocrine growth factors such as endothelin-1 (ET-1) (Venuti et al 1998). ET-1 is a potent vasoconstrictor peptide, secreted by keratinocytes, which influence the growth and function of epidermal and dermal cells by binding to specific growth factor receptor subtypes that belong to the family of G-protein-coupled receptors. Exposure to external stimuli such as UV irridiation leads to upregulation of ET-1 expression (Tsuboi et al 1994), which in turn stimulates melanocyte proliferation and melanogenesis (Tada et al 1998).

1.2.5.3 E5 and 16 K subunit c/ductin

Several viral oncoproteins such as BPV E5 proteins (Goldstein et al 1991), HPV E5 proteins (Conrad et al 1994) and T-cell leukaemia virus type 1 p12 (Franchini et al 1993) bind to 16 KDa. 16 KDa is a highly conserved transmembrane protein which is the subunit c component of the vacuolar H (+)-ATPase (V-ATPase) and a component of the connexon channel of gap junctions. 16 KDa is very hydrophobic and is thought to contain four transmembrane segments arranged as a four alpha helix bundle (Finbow et al 1992). The V-ATPase is one of the most fundamental enzymes in nature. It functions in almost every eukaryotic cell and energizes and acidifies endosomes, Golgi apparatus and other endomembranes compartments (Nelson et al 2000). V-ATPases are multisubunit, heteromeric proteins composed of two structural domains, a peripheral, catalytic V1 domain and a membrane-spanning V0 domain. 16 KDa provides the pathways for proton translocation in theV0 sector of the ATPase. The binding of E5 protein to 16 KDa leads to

impairment of function, inhibition of endosomal acidification in cells transformed by BPV-1 and in cells expressing HPV-16 E5 (Straight et al 1993). PV E5 proteins are not conserved in their aminoacid sequences; however, the HPV-6 and HPV-11 E5 proteins share structural properties with BPV-1 E5 protein. They contain a Cys-X-Cys sequence which is located near the carboxyl terminus and the position 17 (a glutamine residue) within the hydrophobic domain. Both of these regions have been shown to be important for the transforming activity of BPV-1-E5 protein (Horwitz et al 1989). A part of that, the glutamine residue mediates binding of E5 to the 16-KDa vacular ATPase component (Goldstein et al 1992). In HPV-6, there is a binding domain other than the glutamine residue; a construct of HPV-6 E5 lacking the carboxyl terminus and glutamine residue maintains the ability to bind to ductin (Conrad et al 1993). Mutational analysis of HPV E5 proteins demonstrated that the interaction with the V-ATPase is mediated by the central hydrophobic region of the viral proteins, and some E5 mutants able to bind the V-ATPase do not impair V-ATPase function (Adam et al 2000). In BPV-4, both the hydrophilic C-terminal tail and the residue at position 17 in the hydrophobic domain are crucial for E5 functions including binding to ductin and transactivation of cyclin A promoter (O'Brien et al 1999). It has been suggested that induction of transformation in established murine keratinocytes expressing BPV E5 is due to E5-induced malfunction V-ATPase since these cells do not normally express PDGF receptor (Leptak et al 1991).

1.2.5.4 E5 and gap junction

Down-regulation of gap junction-mediated cell-cell communication is another biological activity of PV E5 and observed in keratinocytes expressing HPV-16 E5

and PalF cells expressing BPV-4 E5. Gap junctions are clusters of intercellular channels between adjacent cells, which allow the transfer of ions, metabolites and messenger molecules less than 1 KDa in size between connected cells. The structural proteins comprising these channels, collectively called connexins, are members of a highly related multigene family consisting of at least 13 members. Each connexin has four transmembrane domains and is oriented with the N- and C-termini located in the cytoplasm (Bruzzone et al 1996). It has been demonstrated that HPV-16 E5 induces a significant reduction of gap junctional cellular communication in keratinocytes, which is probably mediated by a dephosphorylation of Cx43, the major component of gap junction (Oelze et al 1995). Recently, it has been reported that BPV-4 E5, the protein analogous to HPV-16 E5, is able to inhibit intercellular communication in PalF cells expressing E5 compared with control cells. This has been examined by injecting a single transformed cell with a fluorescent dye that show no spreading of dye to surrounding cells whereas control cells show spreading of the fluorescent dye to neighbours (Faccini et al 1996). Down-regulation of gap junction has been attributed to binding of E5 to ductin, which comprises a part of the gap junctional complex (Finbow and Pitts 1993). However, binding to ductin is not sufficient for down-regulation of gap junction communication as Ashrafi et al., (2000) have shown that same mutant forms of BPV-4 E5 can not inhibit gap junctions communication despite their ability to bind ductin *in vitro*. Therefore, down-regulation of gap junction can be dissociated from growth in suspension and is not necessary for transformation, although it may help the establishment of a transformed clone. Expression of E5 in the deep layers of papillomas would lead to temporary closure of the gap junction channels and blockage of transduction of

homeostatic control signals exerted by uninfected neighbour cells onto infected cells.

1.2.5.5 E5 and Apoptosis

Papillomavirus depends on the host DNA replication machinery to synthesize its own DNA, since the virus does not encode a DNA polymerase. The host DNA replication machinery is readily available in the proliferating basal cells, but is limited in the postmitotic differentiated cells of the suprabasal compartment. The response of healthy cells to inappropriate DNA replication is apoptosis. Therefore it is to the advantage of the virus to promote DNA replication and cell proliferation, delay terminal differentiation and suppress apoptosis within the host cells (McMurray et al 2001). Apoptosis is the best-charaterized form of programmed cell death and is of fundamental importance in tissue homeostasis. The morphological features of apoptosis consist of chromatin condensation and cell shrinkage whilst the biochemical features include DNA fragmentation, protein cleavage at specific locations, increased mitochondrial membrane permeability (Kerr et al 1972; Kerr et al 1994). In mammalian cells, two major pathways have been identified to be involved in the initiation of apoptosis: the extrinsic death receptor (DR) pathway and the intrinsic mitochondrial pathway. In many cell types including numerous tumor cells, both extrinsic and intrinsic pathways act in coordination, leading to activation of the executioner caspase cascade. Activation of the DR pathway takes place by ligation of members of the tumour necrosis factor (TNF) family, such as Fas ligand (FasL; also called Apo1) and Apo2 ligand (Apo2L; also called TNF-related apoptosis-inducing factor [TRAIL]), to DR on the plasma membrane. TRAIL is a member of the TNF ligand family that rapidly

induces apoptosis in a variety of transformed cell lines. It cross-links with the death receptors DR4 or DR5, while the FAS ligand, which is a type II transmembrane protein that belongs to the TNF family, binds to the Fas CD95 receptor. Binding of these ligands to their respective receptors, leads to aggregation of the receptors. Trimerization of the activated Fas or TRAIL receptors leads to recruitment and binding to the adaptor molecule FADD (Fas-associated DD), and subsequently activation of initiator caspase-8 (Kischkel et al 2000). The complex protein which is formed by binding of Fas and TRAIL to FADD is called death-inducing signalling complex (DISC) which stimulates the activation of procaspase-8. The caspase-8 is member of the interleukin-1beta converting enzyme (ICE) family of cysteine proteases. The activated caspase-8 is released into the cytoplasm and initiates a protease cascade that activates "effector" caspases, such as caspase-3 and caspase-7. Fas and TRAIL receptors are widly expressed on normal epithelial cells. Downregulation of Fas expression is a common abnormality in gynaecological cancer whereas TRAIL receptor is not reduced in cervical cancer. HPV-16 like many other viruses has evolved mechanism to delay apoptosis in the infected cells by preventing intrinsic p53-dependent apoptosis mediated by the expression of E6. Furthermore, it has been reported that HPV-16 E5 impairs extrinsic apoptosis mediated by TRAIL and FasL in human keratinocytes. The expression of E5 reduces the surface expression of Fas receptors, suggesting that a reduction of Fas receptors lowers the probability that ligands reach their respective targets causing E5 cells to be less susceptible to FasL-induced apoptosis. It has also been observed that DISC formation induced by TRAIL is impaired, although, there are no differences in the expression of the death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) between E5 and control cells (Kabsch and Alonso 2002).

Fas-induced apoptosis can be blocked by activation of EGF via EGF-EGFR binding which causes activation of its downstream effector (PI3K)Akt. Akt is known as protein kinase B (PKB) and an important molecule in mammalian cellular signaling as anti-apoptotic and cell survival signals. PKB has been implicated as a major factor in many types of cancer due to its ability to overcome the natural apoptosis signals that the cell receives (Franke et al 1995; Kulik et al 1997). Since HPV-16 E5 can enhance EGFR activation, human keratinocytes expressing HPV-16 E5 were examined for apoptosis following exposure to UVB irradiation. Protection of the cells from UVB-irradiation-induced apoptosis was attributed to expression of E5 which in the presence of EGF, acts to keep the cell survival Akt and ERK1/2 at high levels in UVB-irradiated human keratinocytes (Zhang et al 2002).

1.2.5.6 BPV-4 E5 and cell cycle regulators

The cell cycle in mammalian cells is regulated by the coordinated activity of a family of cyclin-dependent kinases (CDKs). These are positively and negatively regulated by the cyclin and CDK inhibitor families (Sherr and Roberts 1995). Based on the timing of their appearance in the cell cycle, cyclins can be divided into two groups: the mitotic cyclins A and B and the G1 cyclins of the D and E families (Lew et al 1991). There are at least two distinct families of CDK inhibitors: the INK4 and the Cip/Kip inhibitors. Both families play regulatory roles during the G1/S cell cycle check point (Hunter and Pines 1994). The inhibitors of Cip/Kip family which consists of p21, p27 and p57 control the G2 check point as well. The p27 has capacity to arrest cells in G2 (Polyak et al 1994). In response to growth promoting signals, cyclin D is synthesized and forms a complex with cyclin

dependent kinase 4 (CDK4) and CDK6, which provides a mechanistic link between extracellular growth signals and the initiation of entry into S phase from G0/G1. Its role in this pathway is to phosphorylate the members of retinoblastoma Rb family (Ely et al 2005). The phosphorylation of Rb protein inhibits its repressing function, permitting the release and activation of E2F transcription factors, which in turn up-regulates the genes required for S phase, such as cyclin A, cyclin E, c-myc and DNA polymerase (Dyson 1998). Cyclin A promotes both G1/S and G2/M transitions. It is also required for DNA synthesis and once transported into the nucleus, it forms a complex with cdk1, which is required for entry into mitosis (Pagano et al 1992). Deregulation of cell cycle is a common strategy employed by many DNA and RNA viruses to trap and exploit the host cell machinery towards their own benefit. It has been reported that BPV-4 E5 expression in NIH3T3 cells promotes transcriptional activation of cyclin A promoter and increases endogenous protein levels in cells maintained in suspension culture and in low serum, whereas both theses culture conditions promotes down-regulation of cyclin A in control cells (O'Brien and Campo 1998). In addition, cyclin A associated kinase activity is increased in E5-3T3 cells despite elevated levels of CDK inhibitor p27 protein. Continued proliferation of E5-3T3 cells in the presence of p27 has been attributed to the fact that the ability of p27 to block CDKs activity and cell cycle progression might be disrupted by E5 expression. Additionally no correlation was observed between elevated levels of p27 and cell cycle arrest or inhibition of cyclin A-cdk following serum removal.

1.3 Host immune system

The immune system is a group of cells, molecules, and organs that act together to defend the body against foreign invaders that may cause disease, such as bacteria, viruses and parasites. There are two types of immunity: the **innate immune system** and the **adaptive immune system**, which are crucially important in eliminating infections. The innate immunity is characterised by induction of non-specific responses and no memory or lasting protective immunity can be achieved. The innate immune system forms early barriers to viral infection at epithelial borders and is stimulated via several immunomodulatory cytokines and cellular effectors including monocytes, macrophages, natural killer (NK) and antigen presenting cells (APC). Such cells rapidly differentiate into short-lived effector cells whose main role is to get rid of the infection. However, in some cases, the innate immune response is not able to eliminate infection and requires the induction of the adaptive immune response. Adaptive immune responses are activated in peripheral lymphoid organs.They allow for specific recognition and elimination of antigens that are abundant in the body fluids or within cells. Adaptive immunity provides long-term immunity to reinfection, (immunological memory). The major effector cells of the adaptive immune response are B and T lymphocytes. B lymphocytes express and secrete antibodies, which target and neutralise humoral antigens for later destruction, whereas cytotoxic T lymphocytes recognize and destroy infected or malignant cells. T cells react directly against a foreign antigen that is presented to them on the cell-surface of a host. Several changes including high levels of cytokines released by antigen presenting cells (APC) which leads to an interaction between T cell receptors (TCRs) and peptide-MHC class I complex at the interface between the T cell and APC. This binding is

critical for selection of the T cell in the thymus, as well as the activation of mature T cells in the periphery, and complete stimulation and differentiation of T cell into cytolytic effectors $CD8^+$ T is induced from interaction between TCR and peptide/MHC class I on the surface of target cells, leading to killing the infected cells (Gonzalez et al 2005; Kalergis et al 2001; Kalergis 2003)

1.3.1 The major histocompatibility complex

The major histocompatibility complex (MHC) is a multi-gene complex critical to vertebrate immunity. It is contained within about 4 Mbp of DNA (1 % of the genome) on the short arm of chromosome 6 at 6p21.3 (Campbell and Trowsdale 1993). The MHC is the most gene-dense and polymorphic region of the mammalian genome and is associated with resistance to infectious diseases, autoimmunity and transplantation (Kumanovics et al 2003). The MHC genome regions of the human, chicken and mouse have been fully sequenced and mapped, but information on the MHC genome structures of other vertebrates is limited. The human MHC is divided into three distinct nonoverlapping regions designated from the centromere to the telomere as the class II, III and class I regions, respectively. Class I is suggested to be the most divergent region between different animal species, class II is intermediate and the class III region is conserved in gene diversity and organization.

The human MHC is known as human leukocyte antigen (HLA) system, which is distinguished from other regions of the human genome by a high gene density with about 40 % of the expressed genes (class I and class II), with class I having immune system functions and large number of duplicated and polymorphic genes. The human MHC (HLA) class I molecules have a critical function in the

recognition of virally infected cells by the cytotoxic $CD8^+$ T cells. Since CTLs cannot access the interior of the cell for inspection, MHC molecules present intracellular antigens on the surface of nucleated cells, where they are recognized by cytotoxic T lymphocytes (Wong and Pamer 2003). The MHC molecules are extensively polymorphic, which is critically important in antigen recognition by T cells. The MHC polymorphisms confer allelic-specific peptide-binding properties and broaden the repertoire of peptides that can be presented by different species (Adams and Parham 2001; Falk et al 1991). This behaviour of T cells is called MHC restriction. The MHC class II molecules are encoded in HLA-D region, which has three main subregions: HLA-DR, DQ and DP, each of which contains one α and one β gene. An association between HLA α chain and β chain forms heterodimers, with peptide binding grooves on the cell surface of antigen presenting cells such as B cells and macrophages. The cell surface glycoproteins of class II serve as receptors that present exogenously derived peptides to $CD4^+$ helper T cells. The class III genes encode a variety of immune and non-immune system molecules; most of them not involved in antigen presentation and include cytokines and components of the complement system.

1.3.1.1 Major Histocompatibility Complex class I

The major histocompatibility (MHC) class I molecules are subdivided into two families, MHC class Ia or classical, and MHC class Ib or non-classical.

1.3.1.1.1 Classical MHC class I

The classical MHC I molecules are a family of extremely polymorphic cell surface glycoproteins expressed on the cell surface of all nucleated cells. These molecules

play crucial role in warning the immune system against the virally infected and transformed cells by facilitating the immune recognition of the intracellular viral peptides and altered self-proteins (Hewitt and Dugan 2004). The MHC molecules were first demonstrated in mice and called H-2 (H-2K, D and L) antigens. In human MHC are also known as HLA (human-leucocyte-associated antigens) because they were first demonstrated on leucocytes. Human MHC class I locus contains smaller loci for the classical genes, named A, B and C. In bovine, serological analysis of lymphocyte cell provided the first evidence for highly polymorphic MHC genes in cattle and other ruminant species. The MHC of cattle was thus named the bovine leucocyte antigen (BoLA) system, which function in a similar manner to those of mice and humans (Amills et al 1998). The bovine MHC class I (BoLA)-A locus contains (BoLA)-A11, A20, BoLA-HD1, -HD6 and BoLA-HD. MHC class I molecules are composed of a type 1 membrane anchored heavy chain (HC, 44 KDa) and a non-covalently associated soluble protein termed β_2-microglobulin (β_2M) (12 KDa). The heavy chain is composed of three extracellular domains called (α1, α2 and α3) transmembrane and C terminal cytoplasmic domain. The α1 and α2 domains form the peptide-binding site where is a groove on the distal surface of the MHC class I molecule and binds antigenic peptides of 8–10 amino acids in length (Falk and Rotzschke 2002; York et al 2002). The maturation of MHC class I proteins within the endoplasmic reticulum (ER) is regulated by chaperones calnexin and calreticulin (Sadasivan et al 1996). Following cotranslational translocation of MHC class I heavy chain and β_2-microglobulin into ER via the Sec61 complex, both HC and β_2M mature, where HCs dissociate from calnexin and associate with β_2-microglobulin. After proteasomal degradation of unwanted proteins or defective ribosome products,

resulting peptides are translocated into the endoplasmic reticulum by the transporter associated with antigen processing (TAP) (Hengel et al 1997; Koopmann et al 1997). The loading of peptides into MHC class I molecules is regulated by a multi-protein complex in the ER termed the loading complex, which includes TAP, and is a prerequisite for its efficient transport to the cell surface. Misfolded MHC class I molecules or those that are not loaded with peptides and are therefore retained in the ER are retrotranslocated to the cytosol for degredation (Hughes et al 1997; York et al 1994). Following peptide binding, MHC-peptide complex are released from ER and shuttled via the Golgi and vesicular transport to the cell surface where they are scanned by CTLs.

1.3.1.1.2 Non-classical MHC class I

These molecules show homology to classical class I molecules but generally have limited polymorphism, low cell surface expression, and more restricted tissue distribution (Shawar et al 1994). In human, non-classical MHC class I (MHC class Ib) includes HLA-E, HLA-F, and HLA-G molecules. The function of the non-classical MHC class I molecules remains unclear, but some of them may have more specialized antigen presentation activities. HLA-G has been shown to play a role in immune tolerance during pregnancy, in tumour escape from immunosurveillance, and probably in transplantation (Rabreau et al 2000; Rouas-Freiss et al 2000). HLA-E is another non-classical class I molecule and, like classical MHC class I loci, the HLA-E gene is transcribed in many tissues, but little is known about its localisation within the cell. Recent studies have shown that HLA-E is a major ligand for the CD94-NKG2 receptors expressed by natural killer (NK) cells, and is thought to be involved mainly in innate immunity (Borrego et al 1998). The

structure of HLA-E is similar to that of the MHC class Ia molecules, but the groove that is formed between α1 and α2 domains is occupied by the leader peptide derived from amino-acid residues 3–11 of the polymorphic classical HLA-A, HLA-B and HLA-C. The delivery of class I MHC leader peptides to HLA-E is dependent on the presence of the TAP transporter, where they can interact with HLA-E (Braud et al 1998b). This peptide binding is highly specific and stabilizes the HLA-E protein, allowing it to migrate to the cell surface.

1.3.2 Natural killer Cells

Natural killer cells are important contributors to innate immune defences against viral infections. They were first identified by their ability to spontaneously mediate lysis of certain tumour cells and by their large granular lymphocyte morphology (Trinchieri 1989). NK cells express receptor that respond to stimulation by MHC class I on surface of target cells. In human NK cells, there are two types of receptors: the killing inhibitory receptors (KIRs) and the lectin-like heterodimer CD94/NKG2 receptors. The KIRs recognize and bind to self-MHC class I molecules, and alter the balance between the activating and inhibitory signals that regulate NK cell function, thus preventing NK cells from being activated by healthy target cells. In virus infected cells, downregulation of MHC class I lead to activation of NK cells via stimulating activating receptor by tyrosine kinase, and overriding negative signals (Karre et al 1986). The CD94/NKG2 receptors recognize MHC leader sequence peptide presented by the non classical MHC class I molecules such as HLA-E (Braud et al 1998a). These molecules are able to deliver negative signals to NK cells through immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic domain, which acts to recruit tyrosine

phosphatases, thereby cutting positive signal pathways (Eissmann et al 2005; Lanier 1998; Watzl 2003). The activating receptors provide a mechanism by which NK cell function is stimulated. Thus, NK cells would kill classical MHC I negative virus-infected cells, unless non-classical MHC-E molecules are present.

1.3.3 Papillomavirus and immune response

Coexistance of a virus and its immunocompetent host requires a balance between the rates of viral replication and viral clearance by the immune system for mutual survival. The host's immune system employs a variety of strategies to eliminate the virus, whereas the virus has developed an array of immune evasion mechanisms to escape its elimination by the host's immune system. Papillomaviruses, like other small oncogenic DNA viruses, are intracellular pathogens and their genome replication is dependent on the cellular replication machinery. They have evolved mechanisms to avoid immune recognition in order to establish their infection. The mechanisms for viral immune evasion can broadly be divided into three catogories: avoid immune recognition by the humoral immune response, interfere with the functioning of the cellular immune response, and interfere with immune effector functions. Failure of inducing anti-viral immune response during papillomavirus infection is suggested to be due in part to the nature of the virus life cycle. The early genes products (E1, E2) for viral replication and the viral transforming proteins (E5, E6, and E7) are expressed at low levels that may not be detectable by the immune surveillance, although these transforming proteins are immunogenic when administrated as vaccine preparations and induce rejection of transplantable tumour cells in mice (Tindle 1996). Vegetative viral replication and expression of high levels of viral proteins and viral assembly take place in the upper layers of

epithelium. There is no cytolysis or cytopathic death as consequence of viral replication and viral particle release, because the keratinocyte is a cell destined for death far from the sites of the immune response and not accompanied by inflammation, so that there is no obvious signal to alert the immune system. However, even in the absence of viral-induced cytolysis and cell death, PV-infected cells should activate a powerful antiviral defence system, type 1 interferon (IFN-α and and IFN-β) secretion that has antiviral, antiproliferative, immunostimulatory properties, acts as a bridge between innate and adaptive immunity (Le Bon and Tough 2002). IFN-α and IFN-β are secreted by infected cells as cellular responses to virus infection, they destroy viral mRNA, thus inhibiting viral protein expression, increase the expression of MHC class I in most uninfected cells to enhance their resistance to NK attack and they activate NK cells to kill virus infected cells that lack the expression of MHC class I. Most DNA viruses including papillomaviruses have evolved mechanisms for inhibiting IFN synthesis and signalling. It has been shown that high-risk HPV down regulates IFN-α-inducible gene expression and HPV-16 E6 and E7 oncoproteins directly interact with components of IFN signalling pathways (Barnard and McMillan 1999; Ronco et al 1998), and abrogate these pathways. Furthermore, these oncoproteins inhibit dendritic cells activation as consequence of interference with IFN and also E6 and E7 inhibit MIP-3α transcription, resulting in suppression of the migration of immature Langerhans precursor-like cells (Guess and McCance 2005), the only antigen presenting cells that can activate immature T lymphocytes. Viruses also evolved several distinct strategies to avoid killing of infected cells by the host, including the production of caspase inhibitors (Irmler et al 1997). In papillomvirus infection, HPV-16 E6 proteins inhibit apoptosis by inducing

degradation of p53. Apoptosis is a normal event in the maturation of B and T cells, as well as in their effector function of killing infected cells. Engagement of Fas (a member of the tumour necrosis factor receptor family) on immature dandritic cells (DCs) by Fas ligand (FasL) induces maturation of primary DCs. Fas-activated DCs upregulate the expression of the major histocompatibility complex class II, and secrete proinflammatory cytokines (Rescigno et al 2000). Mature DC cells cause activation of naive T cells through antigen presentation, which in turn produce IFNγ. It has been shown that *in vitro*, both IFN-α and IFNγ are able to increase the expression of a number of apoptosis-related proteins including Fas thereby rendering cells susceptible to Fas-mediated apoptosis (Kamei et al 2003; Ossina et al 1997; Ruiz-Ruiz et al 2000).

The clearance of virus infection requires antigen-specific lymphocytes that can recognize the virally infected cells via ligation of their receptors to MHC class I molecules. MHC class I present viral peptides on the surface of infected cells for recognition and elimination by CTL. The virus has evolved mechanisms to interfere with antigen presentation by encoding oncogenic proteins. It been known that most cancers including cervical cancers induced by HPV, are characterized by losing the expression of cell surface MHC class I (Bontkes et al 1998; Brady et al 2000). Losing MHC expression may be a tumour phenotype rather than a direct result of a disruption by viral proteins. However, reduction of MHC class I expression is also observed in benign papillomas (Araibi et al 2004; Bonagura et al 1994; Vambutas et al 2000). Many investigations have been made recently to determine the mechanism of interference with MHC class I pathways by which papillomavirus avoids immune recognition in PV-associated disease. It has been shown that E7 proteins of high risk HPV-16 and 18 are able to repress the MHC

class I heavy chain promoter (Georgopoulos et al 2000). Moreover, HPV-18 E7 proteins induce disruption of MHC class I pathway through repressing the TAP1/LMP2 promoter that regulates the expression of genes that encode the TAP-1 and LMP-2 proteins, which are involved in transport of the peptide/ MHC class I complex to the cell surface (Williams et al 1996).

1.3.4 Viral infection and MHCI

Major histocompatibility complex (MHC) class I molecules present antigenic peptides to CD8-expressing cytotoxic T lymphocytes (CTLs). This antigen recognition system is critically important for immune surveillance against viruses and tumors. A more radical approach is utilized by HCMV to interfere with MHC class I expression on the cell surface, such as withdrawal of peptides or intracellular retention of MHC complex. In HCMV-infected cells expressing US2 or US11, the MHC class I molecules are transported from the ER back into the cytosol, where they are degraded by proteasomes following their deglycosylation (Wiertz et al 1996). Moreover, HCMV US3 protein impairs maturation and intracellular transport of MHC class I heavy chain by the formation of a complex with β_2M-associated class I heavy chain prior to peptide loading in the ER (Jones et al 1996). The murine CMV (MCMV) m4 (a CTL-evasion gene), encodes a glycoprotein (gp34), which is expressed at the cell surface in a complex with MHC class I molecules, thus inhibiting CTLs recognition of infected cells, without blocking MHC class I surface expression (Kavanagh et al 2001). The adenoviral E3-19K gene product is synthesised on membrane-bound ribosomes in the host cell and subsequently translocated in ER and glycosylated. In ER, the viral protein binds to MHC class I heavy chains, inhibiting terminal glycosylation of MHC class

I complex, thereby preventing its translocation to the cell surface (Andersson et al 1985). It has been demonstrated that the human immunodeficiency virus (HIV) Nef protein down-regulates classical MHC I (Williams et al 2002) but does not down-regulate non-classical HLA-E (Cohen et al 1999), which is mostly involved in positive selection of inhibitory receptors on NK cells.

1.3.5 PV E5 protein and MHCI

PVs E5 proteins may contribute to virus persistence and disease pathogenesis by downregulating MHC class I expression. Lack of surface MHC I would have profound consequences for presentation of viral peptides to the immune system. BPV E5 protein, a major transforming protein, causes a down-regulation of surface MHC class I molecules in transformed cells by reducing transcription of the MHC class I heavy chain gene and inducing heavy chain degradation (Ashrafi et al 2002). It also retains MHC class I in the Golgi apparatus and prevents its translocation, resulting in expression of a lower level of the MHC class I molecules on the cell surface (Marchetti et al 2002). It has been shown that HPV-16 E5 protein causes downregulation of MHC class in transformed human keratinocytes by retaining MIIC class I in the Golgi and INF treatment increases transcription of the MHC class I heavy chain gene and releases the Golgi-trapped MHC class I to cells surface (Ashrafi et al 2005).

1.4 Aims of study

Papillomavirus-induced lesions (papillomas) persist for several months to one year before clearance by cell mediated immune response. Persistance of lesions has been suggested to be due to the fact that the host immune system is unaware of

viral infection. It has been reported that E5 proteins of BPV-4 and HPV-16 cause down- regulation of MHC class I, preventing viral peptide presentation on the cell surface for CTL scanning.

Down-regulation or lack of MHC class I expression on the cell surface in virally infected cells, stimulates the activating receptors of NK cells. Thus, NK cells would kill classical MHC I negative virus-infected cells, unless these receptors are engaged with non-classical MHC class I molecules, which initiate inhibitory signals and block NK cells activity.

The overall aim of this project is the analysis of the interaction between E5 and MHC class I.

The individual objectives are:

- To investigate if BPV-4 E5 can down-regulate MHC class I in natural infections (papillomas) as well as it does *in vitro*.

-To determine the mechanism whereby BPV-4 E5 causes a reduction of MHC class I heavy chain protein.

-To determine whether BPV E5 does down-regulate non-classical MHC I on the cell surface.

- To determine whether likewise HPV-16 E5 down-regulates classical but not non-classical MHC class I.

- To determine whether HPV-16 E5 reduces surface expression of MHC class I in CIN biopsies.

Chapter Two

Materials and Methods

2 Materials and Methods

2.1 Materials

2.1.1 Histopathology materials

Paraffin embedded blocks of upper GIT papillomas

Paraffin embedded blocks of CIN

Paraffin embedded blocks of raft culture

2.1.2 Antibodies

-Supplied from Dakocytomation

Swine Anti-rabbit IgG

Rabbit Anti-mouse IgG

Anti- Ki67 Antigen clone Mb-1

-Supplied from Sigma

Anti-FLAG M2 mAb

mAb 4A3 raised against the Golgi protein GM130

Anti mouse IgG FITC conjugated antibody

Horse Radish Proxidase

-Supplier from Serotec

mAb W6/32 raised against HLA/A,B and C

mAb DT9 anti human HLA-C/E (for FACS & IF) (A kind gift from Dr. Veronique Braud, Centre National de la Recherche Scientifique, Sophia Antipolis, France)

-Supplier from abcam®

mAb MEM-E/02 raised against HLA-E

-Cancer Research UK

mAb HC10 against HLA-A2 heavy chain

mAb IL-A88 against bovine MHC class I heavy chain (from Dr Shirley Ellis, Institute of Animal Health, Compton.)

Polyclonal rabbit IgG (274, 275) against C-terminal domain of BPV-4 E5 (available in laboratory)

Polyclonal rabbit IgG (11547, 11823) against a β-galactosidase-BPV-4 E7 fusion protein (available in the laboratory)

Polyclonal rabbit IgG against C-terminus and N-terminus domains of HPV-16 E5 (from Prof Dan DiMaio, Department of Genetics, Yale University School of Medicine, New Haven, USA)

-Scottish Antibody Production Unit, UK

Normal rabbit serum

Normal swine serum

- CalbioChem- Novabiochem corporation San Diego, USA

Anti-actin (Ab-1), mouse monoclonal IgM antibody (clone JLA20).

2-1-3) Antibiotics

-Sigma Chemical Co.Ltd (Dorest, U.K.)

Ampicillin

-Invitrogen

Geneticin (G-418)

CALBIOCHEM

Blasticidin

2.1.4. Bacteriology

-Institute of Comparative Medicine Central Services

L-broth

LB-agar

-Becton Dickinson Labware (Oxford, U.K.)

Falcon 1059 polypropylene tubes

Falcon 2059 polypropylene tubes

-Bibby Sterilin Ltd (Staffordshire, U.K.)

90mm bacteriological petri dish

-Fisons Scientific Equipment (Leicestershire, U.K.)

Glycerol

-Invitrogen Ltd (Paisley, U.K.)

E. coli DH5-α competent cells with SOC medium

-Nunc (Hereford, U.K.)

Sterile disposable inoculating loops

2.1.5 Cell line

Mouse mastocytoma P815 cells

Bovine primary fibroblast PalF cells

2.1.6 Cell Culture materials

-Institute of Comparative Medicine Central Services

Sterile phosphate buffered saline (PBS)

-Becton Dickinson Labware (Oxford, U.K.)

90 mm tissue culture dishes

Falcon 2097 polypropylene tubes

Falcon 2098 polypropylene tubes

Serological plastic pipettes

Sterile Plastipak syringes

6 well plates

24 well plates

96 well plates

Cover glass 22x22mm

Cover glass 13mm

Sterile plastipak syringes

Sterile syringe needles

-Bibby Sterilin Ltd (Staffordshire, U.K.)

Sterile plastic bijoux

Sterile plastic universal containers

-Invitrogen Ltd (Paisley, U.K.)

Dulbecco's Modified Eagle Medium (DMEM), high glucose,

without calcium chloride

RPMI 1640 + L-Glutamine medium

2.5% Trypsin-EDTA (1X)

Foetal Calf Serum (FCS)

L-Glutamine (100X)

Sodium pyruvate MEM 100MM

MEM (100X) Non-essential amino acid

MEM amino acid (50X)

-Nunc (Hereford, U.K.)

Cryotubes

T25, T75 and T175 cm^2 tissue culture flasks

-Gelman Science, Northampton, England

Sterile 0.2 μm acrodisc filters

-GREINER

Gel loading tips

2.1.7 Chemicals and Reagents

-National Diagnostics, USA

Histo-clear

-Cambridge Bioscience

Bodipy® TR Ceramide

-Novagen

Gene Juice® Transfection Reagent

-Amersham International plc (Buckinghamshire, U.K.)

Enhanced Chemiluminescence (ECL-plus) Western detection agent

-Invitrogen Ltd (Paisley, U.K.)

Agarose (ultrapure electrophoresis grade)

Phosphate buffered saline (PBS)

20 X MES SDS running buffer

20 X MOPS SDS running buffer

20 X MOPS SDS transfer buffer

NUPAGE® Antioxidant

NUPAGE® Sample Reducing Agent (10X)

NUPAGE® LDS Sample Buffer (4X)

Tricine Glycin SDS Sample Buffer

Tricine SDS Runing Buffer (10X)

Nu PAGE™ 4-12% Bis-Tris gel

Nu PAGE™ 16% Tricine gel

-Institute of Comparative Medicine

1X TBE buffer

10 X loading buffer (65% Sucrose, 10mM Tris-HCl, 10mM EDTA, 0.3% BPB, Bromophenol Blue up to 10ml dH2O)

- Gibco

HEPES

-Sigma Chemical Co. Ltd (Dorset, U.K.)

Poly-L-Lysin hydrobromide

3,3- Diaminobenzidine (DAB) peroxidase substrate

β-mercaptoethanol

Bicinchonoinic Acid (BCA) solution

Bovine Serum Albumen (BSA)

Copper (II) sulphate (pentahydrate 4% (w/v) solution)

Ethidium bromide

Nonidet P-40 (NP-40)

Phenol:Chloroform:Isoamyl Alcohol (25:24:1 (v/v)

Ponceau S solution

Tween-20 (Polyoxyethylene sorbitan nonolaurate)

Xylene Cyanol

-University of Glasgow (Glasgow, U.K.)

Crude Ethanol

Glycerol

-VWR International (Dorset, U.K.)

DPX Mountant

Hydrogen peroxide H2O2

Acetic acid

Di-sodium hydrogen orthophosphate (anhydrous)

Dimethyl sulfoxide (DMSO)

100% pure ethanol

Ethylene diamine tetraacetate (EDTA) disodium salt

Hydrochloric acid

Magnesium chloride

Methanol

Propan-2-ol

Sodium acetate

Sodium chloride

Sodium dodecyl sulphate (SDS)

Tri-Sodium Citrate

Tris base

2.1.8 Enzymes and Kits

-Dakocytomation

Dakocytomation envision kit

Strept AB Complex/ HRP

- (AMAXA GMBH)

Mouse T cell Nucleofector kit

-Applied Biosystems (Warrington, U.K.)

Amplitaq® DNA polymerase with Geneamp® buffer

Bigdye v3.1 DNA sequencing kit

Hi-Di formamide

TaqMan® EZ RT-PCR® kit

-Invitrogen Ltd (Paisley, U.K.)

The restriction enzymes Hind III, Xba-I, and their respective reaction buffers.

Alkaline phosphatase

T4 DNA ligase

5X ligase buffer

-Qiagen Ltd (Crawley, U.K.)

QIAquick PCR purification kit

QIAprep plasmid maxiprep kit

QIAprep Spin plasmid miniprep kit

QIAquick Gel extraction kit

RNease mini kit

-Gentra Systems, USA

Generation Capture column kits

2.1.9 Molecular Weight Markers

-Invitrogen Ltd (Paisley, U.K.)

1kb ladder

100bp ladder

See-Blue 2 protein markers

2.1.10 Plasmids

pcDNA empty vector carrying the universal immediate early (IE) promoter of cytomegalovirus (CMV) (Invitrogen, Glasgow, UK).

PcI-16E5 expresses the wild type HPV16-E5 protein under the control of the CMV IE promoter.

pc-4E5 expresses BPV-4 E5 protein under the control of immediate early (IE) promoter of cytomegalovirus (CMV) (Invitrogen, Glasgow, UK).

PEGFP-C1 is a eukaryotic expression plasmid for the Green Flourescent Protein (GFP). Expression is driven by the CMV promoter (BD Biosciences Clontech).

pBS/A2 is a plasmid (pBlueScriptII SK(+)) carrying HLA-A2 in EcoRV/SpeI a gift from Barbara Marchetti, Institute of Comparative Medicine, Glasgow (pAL356IILA-A2 gift from Dr Steve Mann from Cardiff, was cut A2 and put in pBlueScriptII SK(+).

pcDNA6/V5-His/*N*50001* expression vector tagged with V5 epitope and carrying bovine non-classical (*N*50001)* gene in HindIII/XbaI, under the control of immediate early (IE) promoter of cytomegalovirus (CMV), (from Dr Shirley Ellis, Institute of Animal Health, Compton).

pcDNA6/V5 His/ HLA-A2 a plasmid carrying HLA-A2 cloned in HindIII/XbaI, was excised from pBlueScriptII SK(+) in HindIII/XbaI sites.

pcDNA6/V5/HLA-E a plasmid carrying HLA-E cloned in HindIII/XbaI, excised from pBlueScriptII SK(+) in KnpI/BamHI sites.

pZipneo empty expression vector of Molony murine leukaemia virus (MoMuLV)

pZip-4E5 expresses BPV-4 E5 protein under the control of MoMuLV long terminal repeat LTR promoter, (available in laboratory).

pFLAG CMV-1 the amino-terminal FLAG fusion expression vector (Sigma), allows transient or stable transfection with neomycin (G418) selection marker.

pFLAG CMV-1/*N*50001* expresses bovine non-classical MHC class I, cloned in HindIII/XbaI sites and under transcriptional control of promoter regulatory region of HCMV.

2.1.11. Miscellaneous

-Amersham International plc (Buckinghamshire, U.K.)

Hyperfilm ECL

-Whatman International Ltd., Maidston, Kent, England

Whatman 1 filter paper

-Applied Biosystems Ltd (Warrington, U.K.)

Real-time PCR 96 well plates and their caps.

-Elkay International (Basingstoke, U.K.)

Microcentrifuge tubes

-Sigma Chemical Co. Ltd (Dorset, U.K.)

Formaldehyde

-Whatman International Ltd (Maidstone, U.K.)

Whatman 3MM filter paper

-Dakocytomation

Pap pen

-VWR International Ltd

Lab-Tek II chamber slide

-Invitrogen life technologies

Lipofectamin Reagent

Plus Reagent

NuPAGE 4x LDS sample buffer

NuPAGE 10x sample reducing agent

NuPAGE Antioxidant

-Roche Diagnostics, Mannheim, Germani

Protease inhibitor cocktail tablets

-vector lab, USA

VECTABOND

-Camlab, Cambridge, England

1.5 ml Screw Top Microtubes

-Morison store

Marvel (Dried skimmed milk)

2.1.12. Water

Distilled water (dH2O) for the preparation of buffer stocks was obtained from a Millipore MilliRo 15 system, and when used for protein, enzyme, RNA or recombinant DNA procedures was further purified on a Millipore MilliQ system to 18MΩ/cm.

Sterile distilled water for making up tissue culture media was supplied by the Institute of Comparative Medicine Technical Service.

2.2Methods

2.2.1 Samples collection

Bovine papillomas from the palate, rumen and oesophagus, as well as samples from palate, tongue and buccal mucosa were collected post-mortem from animals that were referred to the University of Glasgow Veterinary School. Tissue samples were immersed in freshly prepared 10% formaldehyde in phosphate buffer at pH 7.5 in order to preserve tissues from decomposition. After overnight fixation, samples were histologically examined, trimmed for proper size and made ready for processing.

2.2.2 Processing

Fixed tissue samples were placed on small hollow metal cassettes to allow liquids passage and then covered with a lid prior to processing. Tissues were processed automatically in a tissue processor, which moves the tissues around the various agents on a preset time scale. The first process was performed with a series of alcohols (30 % ETOH in ddH2O 1-2 hours, 50% ETOH in ddH2O 1-2 hours, 70 % ETOH in ddH2O 1-2 hours, 95% ETOH 3 hours, 100 %ETOH 1hour) in order to remove water gradually from tissue. The second process was performed with three sets of xylene (30 minutes, 1 hour, 30 minutes) to clear the alcohol. Tissues were transferred from cassettes, orientated on the mould and tissues were embedded with three sets of paraffin in order to protect tissue from collapse after water

removal. The tissues were waxed by pouring molten paraffin on them and then the blocks placed on cold plate at 4°C prior to sectioning.

2.2.3 Raft cultures

Organtypic epithelial raft cultures are tissue culture systems that allow keratinocyte monolayers to stratify and achieve full differentiation via culturing of the cells on collagen gels at the air-liquid interface. The immortalized human keratinocyte HaCaT cells were stably transfected with 4μg pcDNA, pL2, pc16-E5 or pL2 16-E5 per 1x 10^6 cells using Lipofictamine Plus (Invitrogen) according to manufacturer's instructions. The transfected cells were selected in DMEM medium containing 500μg/ml G418 (Invitrogen) for 21 days (Ashrafi *et al.*, 2005). The culture method used for stratification and differentiation of keratinocytes was performed by Dr Sheila Graham, IBLS, Glasgow. The collagen matrix solution was made on ice with concentrated Ham's F12 medium, reconstitution buffer and 3T3 J2 fibroblasts. One millilitre of the collagen matrix solution was poured into 24-well microtiter plates and then equilibrated with 1 ml of growth medium and incubated overnight at 37°C. The E5-transfected HaCaT cells were seeded on the top of the gels and maintained submerged for 24-48 h. The collagen rafts were raised and placed onto stainless steel grids at the interface between air and culture medium. The growth medium was a mixture of F12: Dulbecco's medium supplemented with 0.5 μg/ml hydrocortisone, 10ng/ml EGF, 10% FCS, 1mM/L sodium pyruvate, 2mM/L L-glutamine, 10mM/L HEPES, 5μg/ml insulin, and 5 μg/ml transferrin. HaCaT cells were allowed to stratify for 14 days, the medium being replaced every other day. The cultures were then harvested, fixed in 10% formalin buffer, and embedded in paraffin.

2.2.4 Preparation of slides

Glass slides were cleaned with acetone and immersed in VECTABOND solution (vector lab, USA) for five minutes. The solution was prepared by mixing 7 ml of the stock into 350ml acetone. The slides were then washed in distilled water, air-dried at room temperature.

2.2.5 Sectioning

Wax embedded tissue blocks of papillomas and CIN biopes (kind gift from Dr David Millan, NHS, Glasgow), and Raft culture blocks, were cut on microtome (Shandon Finesse, UK) in serial sections at thickness 1.5μM, then floated on warm water at 40°C to soften and flatten tissue sections. These sections were transferred onto coated slides (Vector lab, USA) to increase adhesion of the sections, which were later subject to heat-mediated antigen retrieval pre-treatment. The sections were allowed to dry overnight at 60°C and stored at room temperature until use.

2.2.6 Immunohistochemistry principles

Immunohistochemistry is a technique which allows detection of antigens in tissue sections by the use of labelled antibodies as specific reagents through antigen-antibody interactions. The resulting antigen-antibody complex can be detected by either direct labelling of the antibody or indirectly by use of a biotinylated secondary antibody. The method undertaken for this evaluation was an indirect labelling technique that utilises a streptavidin-biotin complex to amplify the signal. This complex consists of the unlabelled primary antibody bound to the antigen, which is subsequently bound by the biotinylated secondary antibody. A third layer is used which involves applying a complex of streptavidin and horseradish

peroxidase (HRP). The avidin and biotinylated HRP are mixed at appropriate concentrations allowing a complex to form. The pre-formed complex is then attached to the biotinylated secondary antibody. The culmination of the procedure is the attachment of a marker in order to visualise the complex. The marker used in this study was 3,3 diaminobenzidine tetrahydrochloride which produces a brown end product which is highly insoluble in alcohol and other organic solvents. Potential non-specific antigens were blocked by application of the appropriate unlabelled normal serum.

2.2.7 Preabsorption of BPV-4 E5 antiserum

Preabsorption of the antisera with the respective antigens by using the complementary peptide results in abolition of the staining. This was used to verify the specificity of the immunostaining of BPV-4E5. Typically, 30µl (1mg/ml) of a synthetic E5 peptide representing the C-terminal fragment of BPV-4E5, against which the antiserum was raised, was mixed with 10µl of E5 antiserum in sterile eppendorf tube in 100µl total volume of PBS. The mixture was incubated for 1 hour at 4°C while being continuously stirred. A mixture of E5 antiserum with a L1 peptide instead of the E5 peptide was incubated under the same conditions. The samples were centrifuged at 14,000 rpm for 10 min at 4°C and the supernatant was transferred to new sterile tube.

2.2.8 Antibody optimisation

In immunohistochemistry, the optimum antibody titre may be defined as the highest dilution of an antibody that results in the maximum amount of specific staining with the least amount of non-specific background staining.

The optimum condition for each of the antibodies was determined by incubation of the antibody with the appropriate control tissues containing specific target antigens. Twelve serial sections from each of the control tissues were cut in order to optimise the dilution and staining procedure. Two sets of six serial sections were stained with the designated antibody. A range of dilutions from 1/50, 1/100, 1/500, 1/1000 and 1/5000 was performed. One of sections received no antigenic unmasking pre-treatment; another set underwent a heat-mediated antigen retrieval procedure in a pressure cooker. Each set of sections include a section that was treated exactly as the other sections except that it was incubated with normal serum instead of the primary antibody. This was used as a negative control. The staining was carried out as per immunohistochemistry protocol.

After completion of staining, sections were analysed by light microscopy. Evaluation consisted of looking for the sections that stained the strongest, along with having the lowest level of background interference. The antibody dilution that showed this was considered optimal and was the dilution at which the material being investigated was stained.

2.2.9 Immunohistochemical staining

Sections were deparaffinised in histo-clear (National Diagnostics,USA) for five minutes and rehydrated through absolute alcohol , 80% , 70% for 1min each and then passed in running water. Sections were placed in 0.5% H2O2 in methanol

solution for twenty minutes to block endogenous peroxidase, followed by rinsing in distilled water for five minutes. Antigen retrieval (AR) is an unmasking technique carried out on sections to remove formalin cross-linking with antigenic epitopes. AR was used with the paraffin sections prior to MHC class I and Ki67 staining for seventy five seconds of pressure cooking at fifteen pounds per square inch (PSI), 0.01M Sodium citrate buffer was pre-heated to boiling prior to immersion of sections. The pressure cooker (Prestige,UK) heating was timed from the moment it reached the required operating pressure indicated by a valve at 15 PSI. Sections were rinsed inTween 20 (BDH, UK) 0.01M TBS pH 7.5, slides were wiped and sections circled with Dako pen. Sections were incubated with appropriate 0.1% normal unlabelled serum (Scottish Antibody production unit, UK) diluted in 0.01M TBS for thirty minutes in a humidified chamber. Normal serum was applied to block any non-specific binding by secondary antibody. The excess serum was then removed and the appropriate primary antibody was applied at the optimum dilution. For human and bovine MHC class I staining, sections were incubated for 1.5 h at room temperature with dilution 1:2000 of mAb HC10 and 1:100 of mAb IL-A88 respectively. For HLA-E staining, sections were incubated for 1.5 h at room temperature with dilution 1:100 of mAb MEM-E/02. For BPV-4 E5 staining, sections were incubated with two different E5 antisera raised in rabbit against E5 C-terminus domain; anti E5 (274) at dilution 1:2000 and anti E5 (275) at dilution1:5000. The specificity of E5 staining was verified by incubation of sections with pre-absorbed E5 antisera at 1:10. For HPV-16 E5 staining, sections were incubated with ratio 1:1 of antisera raised in rabbit against HPV-16 E5 C-terminus and E5 N-terminus domains at dilution 1:500 For Ki67 staining, sections were incubated with mAb MIB (Dako) at 1:200. For detection of

BPV-4 E7, papilloma sections were stained with dilution 1:250 of rabbit antisera 11547 and 11823, which were raised against a β-galactosidase-BPV-4 E7 fusion protein. In all experiments, antibodies were diluted in sodium Azide buffer (0.1g of Bovine Serum Albumin (BDII, UK), 0.01g of Sodium Azide (BDH, UK) in 100 ml M TBS (PH7.5). Sections were immersed three times for 5 minutes each in washing buffer (Tween 20 0.01M TBS (pH 7.5) before incubation with secondary antibody. For sections stained for MHC I and Ki67 detection, biotinylated rabbit anti-mouse secondary antibody (Dako, UK) was applied at a standardised dilution 1/200 for 30 minutes at room temperature. Biotinylated swine anti-rabbit (Dako, UK) was applied to sections that were incubated with primary Ab for BPV-4 E5, E7 and HPV-16 E5 detection. After washing as above, sections were incubated with HRP conjugated streptavidin-biotin complex (Dako, UK) for forty-five minutes, as per manufacturer's instructions. Antibody-antigen interaction was visualised by covering sections with 3,3 DAB, used as chromogen with H2O2 as substrate. This forms a stable insoluble complex, which can easily be seen under microscope. The DAB was reconstituted in five ml tap water as per manufacturers instructions, it was observed that final staining was stronger when DAB was reconstituted in tap water than in distilled water because of metal ions contained in tap water that enhance reaction. Incubation with DAB was timed up to five minutes, during that time, sections were microscopically checked to maximise the staining and minimise any background. The sections were then counterstained with Gills haematoxylin and any back ground staining was removed by placing sections in1% acid alcohol for 10 seconds prior to rinsing in Scott's tap water substitute (STWS) to stain the nuclei blue. The sections were dehydrated through three alcohol dilutions, 70%, 80%, absolute, then cleared in histo-clear, transferred into a

fume hood where they were permanently mounted with DPX mountant (BDH) and coverslipped before examination under microscope.

2.2.10 Imaging

Images were captured and analysed using computerized images analyser (Zeiss KS300) through a video camera connected to a light microscope. This system operates on the principle of converting light images from the microscope into a digitalized value of optical density for a given unit of area in the observation field. The entire mounted section was first examined by a light microscope using a x10 objective to allow observation of whole sections. Selected patches were enlarged at high power magnification. Images were digitized using a digital video camera (JVC KY-55B, Imaging Associates, Thame, UK) with a resolution of 150 x 150 of captured area before being converted into micrometers. Images analysis was performed using images analysis software (Zeiss KS300 Release 3.0, Imaging Associates, Thame, UK).

2.2.11 Cell Culture

All cell culture work was performed using strict aseptic techniques inside a laminar flow hood (class II Microbiological Safety Cabinets, Gelaire BSB4). Cells were grown in T175 cm^2 culture flasks with loosened caps to allow for sufficient gas exchange, incubated at 37°C in a dry atmosphere containing 5% (v/v) CO_2 (Nopco Scientific).

2.2.12 Maintenance of cells in culture

The cultured cells were examined daily by observation of the morphology, the colour of the medium and the density of the cells. In suspension culture, cells were grown in 1640 RPM medium supplemented with 10% FCS and fed by dilution into fresh medium. Confluent cells were centrifuged at 1000 rpm for 5 minutes at room temperature and the cell pellet was resuspended in fresh medium and divided into flasks according to the density. Adherent cells that did not need to be divided were fed by replacing the old medium with fresh D-MEM medium supplemented with 1mM Sodium Pyruvate, 2mM L-Glutamine and 10% FCS. In the confluent adherent cultures, the medium was aspirated and the cells washed once with 10 ml phosphate-buffered saline (PBS). The PBS was removed and cells were treated with 3 ml of pre-warmed trypsin solution (0.25% trypsin in 1x PE buffer; PBS with the addition of EDTA to 1 mM). Trypsin-treated cells were incubated at 37°C until the cells had detached from the flask. Complete medium was added and the cell suspension transferred to a sterile universal tube. The cells were centrifuged at 1000rpm for 5 minutes at room temperature. The pellet was then resuspended in fresh growth medium and the cells reseeded in 2 flasks.

2.2.13 Preservation and Storage of the cells

To freeze cell stocks for storage, confluent suspension cultures were centrifuged and confluent adherent cultures were trypsinised and pelleted as described above. In both cases, pellets were resuspended in growth medium containing 10% (v/v) DMSO. DMSO is a cryoprotective agent which acts to lower the freezing point but causes toxicity to cells at room temperature and for this reason all solutions must be chilled. Suspensions were divided into 1 ml aliquots in 1-2 ml Nunc cryotubes

and placed in a polystyrene box. The cells were slowly cooled by storage at -70°C for 4 hours to overnight in order to allow the water to move out of cells before it freezes. The ampoules were then immediately placed in the liquid nitrogen storage bank containing labelled rack until required. Frozen stocks were recovered by removing the ampoules from liquid nitrogen and placing them into a small, covered bucket of water at 37°C. Cells were immediately diluted into 10ml of the appropriate pre-warmed growth medium, centrifuged, resuspended in fresh growth medium and transferred to 175 cm^2 flasks.

2.2.14 Counting cells

The concentration of viable cells was determined using trypan blue dye which stains dead cells dark blue. In 1.5 ml tube, 50 µl of culture suspension was mixed well into 50 µl of trypan blue (Gibco BRL, UK). A cover slip was fixed on a haemocytometer counting chamber and mixing sample was loaded until an area of 1mm x 1mm x 0.1mm depth of chamber was filled. The number of cells in each of the large corner squares was counted under 10 x objective and a mean of these counts was taken. This mean multiplied by 1x 10^4 represented the number of cells per ml.

2.2.15 P815 cells

Mouse mastocytoma P815 cells expressing either bovine classical N*01301or non-classical N*50001 MHC class I were a kind gift from Dr Shirley Ellis (IAH). Either classical or non classical genes were firstly cloned in expression plasmids under the transcriptional control of the universal IE promoter of CMV in pcDNA6 with Blasticidin gene resistance. P815 cells were transfected with bovine MHC

class I genes and then selected in medium containing 10μg/ml Blasticidin for 21 days.

2.2.16 BPV-4 E5 expression vectors

The BPV-4 E5 open reading frame (ORF) from 332 to 458nt of the BPV-4 genome was cloned in two different expression plasmids, either under the transcriptional control of the universal IE promoter of CMV in pcDNA3 or expressed under the control of the Moloney murine leukemia virus in the vector plasmid pZip-neo.

2.2.17 Transfection of P815 cells with BPV-4 E5 gene using Gene Juice

Aliquots of 1x 10^6 P815 cells expressing either bovine classical or non classical MHC class I were placed in 6-well plates contain 3 ml/ well of RPM-1640 medium supplemented with 10 % FCS and the cells were grown overnight at 37°C in 5% CO2. Transfection reagent was prepared with 100 μl RPM-1640 serum-free medium, 3μl Gene Juice for each transfectant. The mixture was spun around on vortex and incubated at room temperature for 5 minutes. 1μg of pcDNA, pZip neo, pc-4 E5 or pZip-4 E5 was added and the transfection reagent was mixed and incubated at room temperature for 15 minutes. In a sterile hood, the cells were transfected with 1μg DNA and the transfection reagent was added as drops to distribute all over the surface of the plate and further distribution of transfection reagent over the cells was made by moving the plates around, forward and backward. Each transfection was performed in duplicate and a negative control for transfection was performed. The plates were incubated overnight at 37°C and then 3 ml of fresh RPM-1640 medium supplemented with 10% FCS was added to each transfectant and re-incubated overnight at 37°C. Transfected and non transfected

cells (control) were centrifuged at 1000 rpm and resuspended in RPM-1640 medium containing 500μg/ml G418 (Invitrogen). The cells were kept under selection for three weeks.

2.2.18 Transfection of P815 cells with BPV-4 E5 gene using Neoclufector

Mouse mastocytoma P815 cells expressing either bovine classical or non classical MHC class I were stably transfected with two different plasmids carrying BPV-4E5 using Nucleofector (Amaxa- Germany) according to manufacturer's instructions. Transfection reagent was first prepared by mixing (2.25ml Mouse T cell Nucleofector solution, 0.5 ml Mouse T cell solution supplement) and left at RT until use. After adding 1ml of component A to 100ml of Mouse T cell Nucleofector medium, each well of 12-well plates was supplied with 1ml of medium, then 10μl of component B. The plate was pre-incubated at 37°C for 30 minutes at least prior to adding transfectants. Aliquots of $1x10^6$ cells were centrifuged at 90X for 10 min, resuspended in 100 μl transfection reagent. After adding 4μg of plasmid DNA, cells were mixed well and transferred to a cuvette before applying to Nucleofector. After introducing DNA, cells were seeded in pre-incubated medium for 48h. Cells were selected in RPM -1640 medium containing G418 for three weeks. Cells transfected with 2.5μg puma-GFP/sample were used as a positive control and microscopically checked 24 hours after tarnsfection.

2.2.19 Transfection of P815 cells with HPV-16 E5 gene

Mouse mastocytoma P815 cells were stably transfected with either universal plasmid carrying empty vector (pcDNA) or carrying HPV-16E5 (pc-16 E5) using Nucleofector (Amaxa- Germany) according to manufacturer's instructions.

Introduction of HPV-16E5 into P815 cells was performed as BPV-4 E5 transfection protocol.

2.2.20 Cloning of HLA-E gene in pcDNA6 plasmid

The HLA-E gene was constructed in pcDNA6 plasmid by PCR-amplifying the cDNA of HLA-E from the pBLuescript II SK (+) plasmid using the following primers: forward primer was made to include some base pairs from pBL plasmid, Hind III site, Kpn I, and base pairs from HLA-E sequence;5'GGGCGAATTGAAGCTTGGTACCGATTCG '3. The reverse primer was designed to contain some base pairs of pBL, XbaI, and BamH I; 5' GGTGGCGGCCGCTCTAGAACTAGTGGATCCA-'3. To amplify HLA-E, 5 ng of pBL/HLA-E was mixed with 100 ng of each forward and reverse primers, with 1x PCR kit buffer, MgCl2, 2 units Taq DNA polymerase and dNTPs (32 μM of each dATP, dGTP, dCTP and dTTP) in a total volume of up to 50 μl dH2O and then aliquoted into 0.5 ml GeneAmp PCR reaction microfuge tubes. The tubes were placed into the PCR machine (PTC-200 Peltier Thermol Cycle) to make PCR products. For PCR amplification, the sample was heated to 95°C for 3 minutes to inactivate DNase and ensure all DNA duplexes were melted, in addition to activating the Taq DNA polymerase. The DNA was then amplified for 28 times as follows: the sample was heated to denature double stranded DNA at 94°C for 30 seconds, 57°C for 30 seconds for annealing and 72°C for 30 seconds to elongate DNA. After completion of the cycles, the reaction was incubated at 72°C for a further 7 minutes to ensure full extension and then cooled to 4°C. After PCR amplification, 5 μl of the sample was analysed by agarose gel electrophoresis by using appropriate marker to check the correct product was amplified. The PCR

product was digested by using restriction enzymes and appropriate reaction buffer and then purified by using PCR purification kit. The pcDNA6 vector was digested with HInd III and Xba-I sites by using appropriate restriction enzymes and reaction buffer according to the manufacturer's instructions. The digestion of the pcDNA6 vector was monitored by using agarose gel electrophoresis and the cut DNA was purified by using gel extraction kit. The HLA-E fragment was ligated into the cut vector with different inserts:vectors ratio, 1:1, 3:1, 1:3 using 1 µl (4 Units) of T4 DNA ligase in 1x ligase buffer in a 15 µl reaction volume The best ratio of inserts:vectors was 3:1 for ligation. 5 µl of the ligated samples was transformed in *E. coli* DH5-α competent cells. 50 µl of transformation mixes were spread on LB-agar plates containing the appropriate antibiotic (100 µg/ml Ampicillin) and incubated at 37°C overnight. A single bacterial colony was used to inoculate 5 ml L-broth medium containing the appropriate antibiotic in Bijoux tubes and grown overnight at 37°C (to select 10 colonies in 10 Bijoux tubes). Plasmid DNA was prepared from the colony using the QIAprep Spin plasmid miniprep kit according to the manufacturer's instructions. The plasmid DNA was sequenced and transfected into either P815-pcDNA or P815-pc16E5 cells.

2.2.21 Cloning of HLA-A2 gene in pcDNA6 plasmid

HLA-A2 gene was excised from pBluescript II SK (+) plasmid by digestion of 10 µg of pBL vector in appropriate restriction enzymes and buffer in total volume 100 µl of DW. The mixture was incubated at 37°C for 3 hours and then run on 1 % agarose gel. The digested HLA-A2 fragment was purified by using gel extraction kit. The pcDNA6 was digested with Hind III and Xba-I sites by using appropriate restriction enzymes and reaction buffer according to the manufacturer instructions.

The digestion of the pcDNA6 vector was monitored by using agarose gel electrophoresis and the cut DNA was purified by using gel extraction kit. The HLA-A2 fragment was ligated into the cut vector with different inserts:vectors ratio, 1:1, 3:1, 1:3 using 1 µl (4 Units) of T4 DNA ligase in 1x ligase buffer in a 15 µl reaction as bove. After plasmid preparation and DNA sequence, pcDNA6/HLA-A2 plasmid was transfected into either P815-pcDNA or P815-pc16E5 cells.

2.2.22 Cloning of *N*50001* in pFLAG

Bovine non-classical MHC class I heavy chain was excised from pcDNA6 by digestion of 10 µg of pcDNA6/*N*50001* with appropriate restriction enzymes and buffer in 100 µl reaction volume and incubated for 3hours at 37°C. After gel electrophoresis, the *N*50001* fragment was purified by using gel extraction kit. The pFLAG vector (Sigma) was digested with Hind III and Xba-I and reaction buffer according to the manufacturer instructions. The digestion of the pcFLAG vector was monitored by using agarose gel electrophoresis and the cut DNA was purified by using gel extraction kit. The HD59 fragment was ligated into the cut vector with different inserts:vectors ratio, 1:1, 3:1, 1:3 using 1 µl (4 Units) of T4 DNA ligase in 1x ligase buffer in a 15 µl reaction volume as above.

2.2.23 Restriction Enzyme Digestion of DNA

Restriction digests were carried out in small reaction volumes using enzymes and their appropriate concentrated buffer solutions according to the manufacturer's instructions. Plasmid DNA was incubated with 0.5-1µl (5-10 units) enzyme/µg DNA in a compatible buffered solution. Any possibility of inhibition of digestion by enzyme storage buffer was avoided by ensuring that the total volume of enzyme

added did not exceeded 10% of the final reaction volume. Small quantities of plasmid DNA (<5μg) were routinely digested in a 20μl reaction volume as specified by the manufacturer for 1 hour at 37°C. Large digests were carried out in proportionally larger reaction volumes for 5 hours at 37°C. The digestion fragments were analysed by agarose gel electrophoresis as described below.

2.2.24 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed using a horizontal gel cast apparatus (Biorad). Generally, 1% (w/v) agarose gels were used, but small fragments (100-400bp) were separated on 2-4% gels. In order to isolate and purify required DNA restriction fragments, 1% concentration of low melting point agarose was dissolved in 0.5 × TBE buffer (5x TBE : 40 mM Tris base, 16 mM acetic acid, 1mM EDTA, pH 8.0). The gel mix was heated in a glass conical flask in a microwave oven until the particles of agarose gel had completely dissolved. To enable fluorescent visualization of the DNA fragments under UV light, 5% of ethidium bromide (10 mg/ml, Sigma) was added when the gel was hand hot and mixed well. The gel cast apparatus was assembled with a comb containing the appropriate number and size of teeth to form the sample wells and the gel was poured. The comb was removed from the solid gel and the gel placed in a tank, then 0.5x TBE buffer was added until the gel was covered by approximately 3mm. The samples in 1 × loading buffer were loaded in each well along with an appropriate size marker (e.g. 100b ladder, 1Kb ladder) into the first and/or last well in the gel and run at 70-100 constant voltage usually until the samples' blue dye front was 1-3 cm from the end of the gel. The separated DNA was visualised by

illumination with short wave (312nm) UV light and photographed through a red filter onto video print paper using an UVP gel documentation system.

2.2.25 Isolation and Purification of DNA Restriction Fragment from Agarose Gels

The DNA fragment to be used for cloning was recovered from low melting point agarose gel and visualised as described. Under UV light, the fragment was excised from the gel with a clean scalpel blade and the gel slice placed in an eppendorf tube. The DNA fragment of interest was extracted from the agarose gel using a Qiagen Qiaquick gel extraction kit following the manufacturer's instructions.

2.2.26 Ligation of DNA Fragments

DNA ligations are performed by incubating DNA fragments with appropriately linearized cloning vector in the presence of buffer and T4 DNA ligase. To prevent re-ligation, the 5' phosphate residues of linearised vector DNA were dephosphorylated by adding 1μ (1 unit) Calf Intestine Alkaline Phosphatase (CIAP). The reaction mixture was incubated for 1 hour at 37°C followed by a second incubation at 70°C for 10 minutes to stop all enzyme activity. The plasmid DNA and DNA fragments were purified using a Qiagen Qiaquick PCR purification kit. Quantification of the cut vector and DNA fragment was performed by running 1μl of the vector, 2μl of DNA fragment and 2μg φx174 RF DNA on 1.5% agarose gel. Ligation reactions were performed are 1:3 vector: insert. The DNA fragment was ligated into the cut vector using 1μl (1unit) of T4 DNA ligase (Invitrogen) in 1x ligase buffer in a 20 μl reaction volume. This was carried out according to the manufacturer's instructions. An excess of DNA fragment compared to vector and a

negative control (vector without DNA fragment) were used for ligation reactions. Reactions were routinely incubated overnight at 16°C.

2.2.27 Transformation of Competent Bacterial Cells

Plasmids were propagated in commercially available *E. coli* DH5α competent cells supplied as frozen stocks (Invitrogen) stored at -70°C until use. Transformation of bacterial cells was performed in accordance with manufacturer's instructions. Competent cells were thawed slowly on ice, and 20µl of aliquots placed into prechilled 1.5ml tubes. 10µl of ligation reaction or 5ng of the appropriate plasmid DNA was added and gently dispersed with bacterial cells. The cells were then incubated on ice for 30 minutes before being heat shocked for 45 seconds at 42°C. The tube was then immediately placed on ice for 3 minutes. The transformed cells were then supplemented with 180 µl of room temperature SOC Medium (25 bactotryptone, 0.5% yeast extract, 10% mM NaCl, 2.5% mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20mM glucose) to each transformation reaction. The tubes were then transferred to a shaking 37°C incubator (approximately 225rpm) for 1 hour to allow expression of the antibiotic resistant marker. The transformation mixes were spread on L-agar plates containing the appropriate antibiotic, plates were inverted and incubated overnight at 37°C to allow colony formation.

2.2.28 Glycerol Stocks

The transformed bacterial cells were stored as glycerol stocks for future retrieval. 800µl of an overnight culture was mixed gently in 80% sterile glycerol in a 1.5 ml Nunc Cryotubes and stored at -70°C. A sterile yellow pipette tip was used to scrape the top layer of frozen cells when growing up from glycerol stocks required.

2.2.29 Small Scale Preparation of Plasmid DNA (Miniprep)

Small amounts of plasmid DNA were obtained from transformed bacterial colonies to allow the identification of positive transformants. A single colony from streaked selected plates was picked up using a sterile yellow pipette tip, inoculated in 5ml culture of L-Broth (1% w/v Bactotryptone, 0.5% w/v yeast extract, 1% w/v NaCl) containing antibiotic (50μg/ml Ampicillin) and incubated at 37°C with vigorous shaking overnight. 10 separate colonies were generally picked for screening at any one time. 1.5 ml of bacterial culture was pelleted by centrifugation in a microfuge (14 000 rpm, 10 min). Plasmid DNA was prepared from the colony using the QIAprep Spin plasmid miniprep kit according to manufacturer's instructions.

2.2.30 Determination of ligation by restriction enzymes

The presence of ligated DNA fragment was determined by incubation 10 of μl of plasmid miniprep with appropriate enzymes and buffers at 37°C for 3 hours. The digested and undigested samples were run on 1% agarose gel and separated DNA fragments were visualised and photographed as described.

2.2.31 Large Scale Preparation of Plasmid DNA (Maxiprep)

One colony of the overnight bacterial culture was used to inoculate 200 ml of L-broth containing the appropriate antibiotic in 500 ml glass conical flask. The culture was placed in incubator shaker at 37°C overnight. Bacterial cells were transferred in a Beckman tube and centrifuged at 6000g, 10 min, 4°C using a Beckman J2-21 centrifuge. The bacterial pellet was resuspended in 10 ml P1(50 mM Tris-Cl (pH8.0), 10 mM EDTA, 100μg/ml RNase A) and transferred to a

medium centrifuge tube (JA-20), then gently mixed with 10 ml P2 (200mM NaOH, 1% SDS (w/v) and left for 5 minutes at room temperature to ensure efficient lysis. The mixture was neutralized by the addition of 10 ml of ice cold P3 (3 M potassium acetate pH5.5) and the contents were mixed by inverting the tube vigorously several times. The solution was incubated on ice for 30 minutes and then centrifuged at 13 rpm for 30 min at 4°C. The supernatant was filtered through nylon gauze and loaded onto a QIAfilter Mega Cartridge and bacterial debris, formed in a tight pellet at the bottom of the tube, was discarded. The column was washed twice with 30ml of QC (1M NaCl, 50mM MOPS pH7, 15% isopropanol (v/v), 0.15% Triton (v/v)). The plasmid DNA was eluted by 15ml of QF (1.25M NaCl, 50mM Tris-Cl pH 8.5, 15% isopropanol (v/v)). DNA was precipitated by the addition of 10.5ml isopropanol. Nucleic acid was pelleted by centrifugation at 12000 rpm for 30 minutes, the pellet was marked and the supernatant carefully discarded. The pellet was washed with 1 ml 70% ethanol (v/v), transferred to a micro-centrifuge tube and pelleted by centrifugation at 14000 rpm for 10 minutes. After removing the supernatant, the pellet was resuspended in 400 µl dH_2O and DNA quantification was determined as described.

2.2.32 Polymerase Chain Reaction (PCR)

All reagents were provided in the GeneAmp® PCR core Reagents kit purchased from Applied Biosystems. Primer sequences are described in table1. PCR reaction containing 1ng of DNA template, 20pmol of each primer, 200µM each dNTP, 3mM $MgCl_2$ (25mM $Mn(OAC)_2$, 0.5µl (2.5 units) AmpliTaq® DNA polymerase in a total volume of 50µl containing 1x PCR buffer was pipetted into a 0.5ml GeneAmp PCR reaction microfuge tube. A negative control containing no template

and a positive control were always included with each set of PCR reactions. PCR amplification was carried out using a MJ Reasearch PTC-200 gradient cycler. Samples were heated to 94°C for 1 min (denaturing step), 50°C for 1 min (annealing step) and 72°C for 1 min (elongation step). This cycle of denaturing, annealing and elongation was repeated 25 times. The amplified product was determined by gel electrophoresis of 10 µl of PCR reaction.

Table 2: Oligonucleotide PCR primers

BPV-4 E5 Forward	5' CCA TAC GAT GTT CCA GAT TAC GCT 3'
BPV-4 E5 Reverse	5' CCA TCC ATC TAA CCG AGT AAT AGT 3'

2.2.33 DNA sequencing

The correct orientation of nucleotides sequence in all new plasmids was determined using Taq terminator sequencing on an Applied Biosystems Prism 3100 Genetic Analyzer DNA sequencer. The DNA of interest to be sequenced was first prepared as a single strand and underwent PCR amplification. Reaction containing 0.5 µg plasmid DNA, 3.2pmoles of the appropriate primer, 2µl 5x sequencing buffer and 4µl of BigDye™ Terminator Ready Reaction Premix (Applied Biosystems) were made up with distilled H_2O to a total reaction volume of 20µl. 250 µl thin eppendorf tubes were used for all sequencing PCR reactions. The samples were placed in a MJ Research PTC-200 Peltier Thermal Cycler and exposed to 25 cycles of 96°C for 10seconds, 50°C for 5 seconds and 60°C for 4

minutes. The PCR products were purified using PERFORMA® DTR Gel Filtration Cartridges. The columns were first spun at 3000rpm for 2 minutes to remove buffer. The columns were then transferred to sample collection tubes. The PCR sequencing reaction mix was then loaded onto the centre of the gel column. The column was spun for 3000rpm for 2 minutes. The sample was then dried under vacuum for 15-20 minutes and then resuspended in 25µl formamide.

The Samples were loaded and run on the ABI PRISM® 3100 Genetic Analyzer (PE Applied Bio systems, UK), under standard sequencing conditions for generation of automated sequence data. The ABI PRISM® 3100 Genetic Analyzer is a multi-colour fluorescence-based DNA analysis system using the proven technology of capillary electrophoresis with 16 capillaries operating in parallel. The Genetic Analyzer is fully automated from sample loading to data analysis. The sequence was evaluated by examination of chromatogram output using Chromas software.

Table 3: Oligonucleotides used for sequencing

Primer	Nucleotide Sequence	Plasmid Sequenced
Forward	5'GTC TAT ATA AGC AGA GCT CGT TTA GTC AAC 3'	pFLAG-CMV1
Reverse	5'CTG GTG GGC ACT GGA GTG GCT ACT TCC AGG 3'	pFLAG-CMV1
T7 forward	5'ATT AAT ACG ACT CAC TAT AGG GA 3'	pcDNA6
BGH reverse	5'CTA GAA GGA ACA GTC GAG GC 3'	pcDNA6

2.2.34 DNA preparation from P815 cells

For DNA extraction, P815 cells were grown until 80% confluent and washed twice with PBS. The cell pellet was resuspended in 500 µl PBS and then applied to the Capture column by using Generation Capture column kits (Gentra Systems, USA). After quick centrifugation, the cells were washed twice with 500 µl purification solution and quickly centrifuged (2-3 seconds). Next, the cells were washed once with elution solution and pelleted by a quick centrifugation. 200 µl of elution solution was added again and the top of the tubes was wrapped with parafilm and the tubes were put in boiled water (99°C) for 10 minutes and then spun down as

above. After quantification, DNA was amplified and the PCR products were electrophoresed in order to determine the fragment of BPV-4E5 gene.

2.2.35 RNA preparation

RNA extraction from P815 cells was carried out using a Qiagen RNeasy Kit with QIAshredder columns. Cells (maximum 1x 10^7) were used, centrifuged and washed twice with 1x ice-cold PBS. The cell pellet was lysed by addition of 600µl of RTL (10µl 2-Mercaptoethanol, 1ml RTL buffer) and mixed well using vortex. After adding 600µl of 70% ethanol, the lysate was transferred to QIAshredder spin column then centrifuged to homogenise the sample at 8-10x 10^3 rpm for 15 seconds. The flow through was added to a RNeasy mini column and centrifuged as above. Subsequently, contaminants were removed by washing the column with 700µl of RW1 once, and with 500µl RPE twice. The purified RNA was eluted from the column in 30µl of RNase-free water.

2.2.36 Purification of RNA

RNA extract was treated with DNase to remove residual amounts of DNA in order to optimise the efficiency of TaqMan RT-PCR analysis. Typically, 1µg of RNA extract was added to 1µl (1 unit) DNase I Amplification Grade, 1µl of 10x DNase I reaction buffer and the reaction made up to 10µl of DEPC-treated water. After incubation for 15 minutes at room temperature, the DNase I was inactivated by the addition of 1µl of 25 mM EDTA solution to the reaction mixture. RNA sample was heated for 10 minutes at 65°C prior to reverse transcription.

2.2.37 Quantification of Nucleic Acids

The concentration of nucleic acid in a solution was determined spectrophotometrically in a WPA UV1101 Biotech spectrophotometer. Samples were diluted in dH_2O and transferred to a quartz cuvette with a pathway of 1cm. The spectrophotometer was initially calibrated using dH_2O only as a blank. The optical density reading were obtained at 260nm and 280nm; an O.D. reading of 1 at 260nm (A_{260} = 1) corresponds approximately to a concentration of 50μg/ml of double stranded DNA, for oligonucleotides an A_{260} reading of 1 was taken to correspond to ~35μg/ml, and for RNA an A_{260} readingof 1 was taken to correspond to 40μg/ml. The ratio between readings at 260nm and 280nm (A_{260}:A_{280}) provided an estimate of the sample purity; a ratio of ~1.8 indicated that preparations contained essentially no protein or phenol contamination.

2.2.38 Real-time PCR detection of mRNA quantity

Probes and primers for BPV-4 E5, HPV16 E5 and mouse β-actin were designed using the primer Express Software programme (Perkin-Elmer) and conditions used as per manufacturer instruction. Real-time RT-PCR was performed using TaqMan® EZ RT-PCR kit (Applied Biosystems) and PCR conditions were as per manufacturer instructions. Typically, final concentrations of 1x RT buffer; 3mM MnAc; 300μM dATP, dCTP, dGTP; 600μM dUTP; 200nM of each primer; 100nM probe and 5U rTth polymerase were made for each reaction. Each reaction was performed in triplicate using 100ng of RNA. PCR reactions were performed on a sequence detection system (ABI prism 7700, Applied Biosystems) according to the manufacturer's instructions by using universal PCR conditions (60°C for 30 min, 95°C for 10 min, then 94°C for 20 sec and 62°C for 1 min, in 40 cycles).

Standard curves were generated using 10 serial dilutions of 100pg to 10^{-3} pg of each template DNA, which were used to quantify the relative levels of E5 and β-actin mRNA. E5 mRNA levels were normalized to the β-actin controls.

Table 4: Oligonucleotides used for Real Time RT-PCR

primers	Nucleotide Sequence
BPV-4E5 Forward	5' TGT CTT TGT GGC TTA TCT ATG TTT TGT 3'
BPV-4E5 Reverse	5'CCG AGT AAT AGT AGA AAT TAA CAG AAG GTA CAC 3'
4E5 FAM/TAMRA probe	5'CTT TTC TGG TGT GCT TTT AAT TTT CTT GCA CTG TTA 3'
HPV-16 E5 Forward	5' TGA CAA ATC TTG ATA CTG CAT CCA 3'
HPV-16 E5 Reverse	5' CTG CTG TTA TCC ACA ATA GTA ATA CCA ATA 3'
HPV-16 E5 Probe	5' AAC ATT ACT GGC GTG CTT TTT GCT TTG CT 3'
Mouse β actin Forward	5' GCT CTG GCT CCT AGC ACC AT 3'
Mouse β actin Reverse	5' GCC ACC GAT CCA CAC AGA GT 3'
Mouse β actin Probe	5'AAG ATC ATT GCT CCT CCT GAG CGA AAG 3'

2.2.39 Transient Transfection of PalF cells

PalF-4E5 cells were transiently transfected using LIPOFECTAMINE PLUS™ Reagent (Invitrogen) according to the manufacturer's instructions. Subconfluent cells were trypsinised, resuspended in growth medium and aliquots of 1x 10^4 cells/ well were plated in 24-well plate containing 13mm round cover slips. After overnight incubation, the medium was replaced by adding 200µl/well serum-free D-MEM Medium. For each sample, construct of 0.5µg of either pFlag-*N*50001* (bovine non-classical MHC I) or GFP-B275 (human classical MHC I) was diluted in 25µl D.MEM Medium without serum and mixed before adding 1µl PLUS Reagent. In another eppendorf tube, 1µl Lipofectamine Reagent was mixed in 25µl Medium without serum. Following incubation for 15 min at room temperature, diluted DNA and Lipofectamine were combined, incubated as above and transfected into the cells. After incubation at 37°C at 5% CO_2 for 3 hours, 250µl of medium containing 20% FCS was added to transfectant to bring the concentration of FCS to normal (10%) in total volume 500µl. 24 hours later, the medium was removed and the transfected cells were grown in fresh growth medium for 24 hours before staining.

2.2.40 FACS analysis for MHC class I expression in P815 cells

P815- N*01301 and P815- N*50001 cells either expressing BPV-4E5 or carrying empty vector and P815/HLA-A2 or P815/HLA-E expressing HPV16E5 or carrying empty vector were grown in T175 cm^2 flasks until subconfluent. Following centrifugation, cell pellet was washed once with PBS and resuspended in PBS/1% bovine serum albumin at a concentration of 10^6cells/ml. For the detection of surface expression of bovine MHC class I molecules, aliquot of 10^6

cells were incubated for 1h at 4°C with 1: 10 dilution monoclonal Antibody (mAb) IL A88 in 1%BSA in PBS. For detection of HLA-A2, aliquot of 10^6 cells suspended in 100 µl PBS were incubated for 1h at 4°C with 1: 100 dilution of monoclonal antibody W6/32 Ab, (Serotec), in 1%BSA in PBS. For detection of HLA-E, aliquot of 10^6 cells suspended in 100 µl PBS were incubated for 1h at 4°C with anti-HLA-C/E mAb DT9 (1:50; a kind gift from Dr. Veronique Braud, Centre National de la Recherche Scientifique, Sophia Antipolis, France). For detection of mouse MHC I ($H2D^d$), aliquot of 10^6 cells suspended in 100 µl PBS were incubated for 1h at 4°C with 1:80 dilution of anti- $H2D^d$ mAb (Cederlane). After washing twice with PBS, cells were incubated with 1:100 dilution of goat antimouse IgG-FITC (Sigma, St.Louis, Mo) for 30 min at 4°C in the dark. The cells were then washed as above, resuspended in 500 µl PBS and analysed by flow cytometry. If the flow cytometry analysis was not carried out immediately, the cells were resuspended in 500 µl of 3% paraformaldehyde in PBS and kept at 4°C. P815 cells not expressing bovine or human MHC class I were used as a negative control. Cells incubated with secondary antibody were also used as control. For the detection of intracellular MHC class I, firstly, the cells were permeabilized with 0.1% saponin in PBS-B for 30 min at RT. Secondly; the cells were then incubated with primary antibody as described above. All samples were examined in a Beckman coulter EPICS Elite analyzer equipped with an ion argon laser with 15mV of excitation at 488 nm. Data were analyzed using Expo 2 softwere.

2.2.41 Immunofluorescence detection of MHC class I in PalF-4E5 cells

In all experiments, PalF cells (1x 10^4) were aliquoted into 24-well plate containing coverslips and grown overnight. After removal of the medium, cells were washed twice with PBS and fixed in 5% formaldehyde in PBS containing 2% sucrose for 10 minutes at RT. After that time, cells were washed twice and incubated in permeabilizing solution (0.5% NP-40, 10% sucrose in PBS) for 10 minutes at RT and then washed twice as above. To analyze the localization of non-classical MHC class I in BPV 4E5- expressing cells, fixed and permeabilized cells were first incubated with dilution 1:1000 BODIPY ceramide texas red (Invitrogen), (2.5% Hepes in serum-free D MEM Medium) for 30 minutes at 4°C. After removal of BODIPY, cells were incubated in 2.5% Hepes in serum-free D MEM Medium for 30 min at 37°C, followed by two washes in PBS. For detection of bovine non classical MHC class I, cells were incubated with 1:2000 dilution of Anti-Flag® M2 Monoclonal Antibody (Sigma) in PBS containing 5% BSA. After washing twice in PBS, cells were then incubated with 1:1000 dilution of antimouse FITC (Sigma), for 1 hour in the dark. To determine whether the C-terminal truncated BPV-4E5 (4E5T) protein still preserves its ability to down regulate MHC class I, PalF cells expressing either 4E5T or E7 were incubated with a dilution 1:10 mAb IL-A88 in 1% PBS for 1 hour. To analyze the localization of classical MHC class I in E5-expressing cells, construct of GFPB2705 was transfected into PalF cells either express E5 or E7. 48h later, cells were fixed, permeabilized and incubated with BODIPY ceramide for Golgi staining as described above. Following incubation with BODIPY ceramide, cells were washed 3 times and the cover slips were mounted onto slides using Citifluor.

2.2.42 Immunofluorescence detection of MHC class I in P815 cells

As these cells grow in suspension, an eight-well slide (Chamber slides lab-tek, VWR international) was washed with alcohol, air dried and treated with 40µl/well poly-L Lysine hydrobromide (Sigma) for 10 minutes to allow cell adhesion. After removal of poly-l lysine, the slide was washed with distilled water and air-dried. Following centrifugation, cells were washed twice in PBS and resuspended in fixing solution (5% formaldehyde in PBS containing 2% sucrose) for 10 minutes. After two washes, cells resuspended in PBS containing 1% FCS, 2x 10^4 cells/well were dispersed into the wells and left for 15 minutes to settle down. After removal of PBS, cells were covered in permeabilizing solution (0.01% Tween-20 in PBS) for 10 min. After two washes, cells were incubated with 1:1000 dilution of BODIPY ceramide (2.5% Hepes in serum free 1640 RPM Medium) for 30 min at 4°C. After removal of BODIPY, cells were incubated in 2.5% Hepes in serum-free 1640 RPM Medium for 30 min at 37°C. For localization of bovine MHC class I, cells were first incubated with 1:10 dilution of IL-A88 antibody. For localization of HLA-A2, cells were incubated with 1:50 of mAb W6/32. For localization of non classical HLA-E, cells were incubated with anti-HLA-C/E mAb DT9 at 1:50. For localization of mouse MHC I, cells were incubated with anti-$H2D^d$ mAb at 1:80. All cell lines were incubated with primary antibodies for 1h at RT followed by incubation with 1:1000 dilution of antimouse IgG-FITC for 1h in the dark with two washes in between. After that time, cells were washed three times and the slide was mounted with 60mm coverslip using Citifluor.

2.2.43 Imaging

Images were captured using a Leica TCS SP2 true confocal scanner roscope (Leica Microsystems, Heidelberg, Germany) and a wavelength of 488 nm (MHC class I) or 543 nm (GA). The merge between the FITIC and TRITC; GFP and TRITC fluorescent signal was accomplished using the Leica TCS SP2 accompanying software.

2.2.44 Protein extracts preparation

Aliquots of confluent P815 cells grown in 1640RPM medium were centrifuged at 1000 rpm for 5 min and washed two times with 5ml of ice cold PBS. The cell pellet was then resuspended in 1ml of ice cold PBS, transferred to a 1.5ml eppendorf tube and pelleted. The pellet was vigorously resuspended in 300µl of lysis buffer (0.5% NP40, 50mM Tris PH 7.8, 150mM NaCl with a protease inhibitor cocktail (Roche, Lewes, UK) dissolved in the lysis buffer). Following incubation on ice for 30 min, the extracts were then centrifuged in a refrigerated microfuge for 10 minutes at maximum speed at 4°C. The supernatant was transferred to another tube and the cell debris discarded. The protein concentration was quantified using the BCA/$CuSO_4$ assay. For determination of the stability of MHC class I heavy chain protein in BPV-4E5-expressing cells, aliquots of 2x 10^6 E5 or empty vector transfected cells, expressing either bovine classical or non-classical MHC I, were treated with 100µl/ml Cyclohexamide (Sigma) and incubated at 37°C, 5% CO_2. As DMSO is the solvent for cyclohexamide, half of the cells were treated with 1µl/ml DMSO (Sigma) alone. The treated cells were then harvested after 1h, 2h, 5h, 10h and 24h, washed two times with ice cold PBS,

pelleted and kept at-70°C. The pellets were resuspended in 100µl/pellet lysis buffer as described above.

2.2.45 SDS-Polyacrylamide Gel Electrophoresis

The gels used to separate protein were NuPAGE® 4-12% Bis-Tris-HCl buffered (pH 6.4) poly-acrylamide gels purchased from Invitrogen Ltd. Protein samples were mixed with 4x NuPAGE® LDS sample buffer (40% Glycerol, 500mM Tris-HCl (pH 6.8), 8% SDS, 0.075% Serva blue G250, 0.025% phenol Red), and 10x NuPAGE® Reducing Agent (0.5M DTT), at a ratio of 40:10:4 for sample:loading buffer:reducing agent respectively. Following mixing, samples were heated at 70°C for 10 minutes prior to loading. The gel tanks used to separate the proteins were the Xcell Surrlok Mini-cell tanks purchased from Invitrogen Ltd and the electrophoresis was performed as per manufacturer's instructions. The gel was uncovered, placed in tank to allow the migration of samples towards the positive pole and the lower chamber was filled with 20x MES SDS Running buffer (1M MES, 1M Tris Base, 69.3mM SDS and 20.5mM EDTA) (purchased from Invitrogen Ltd). After filling the upper chamber with 200ml MES SDS containing 500µl Antioxidant (Invitrogen), each sample was loaded into the well of the gel with at least one well contains the See-Blue coloured protein marker (Invitrogen). The electrophoresis was performed in MES SDS Running buffer at a constant voltage 200V for 60 minutes.

2.2.46 Electrophoretic Transfer of proteins

Separated protein samples were transferred to nitrocellulose membranes by wet electrophoretic transfer using a Xcell II™ blotting apparatus (Invitrogen). Blotting

protocol was as per manufacturer's instructions. 20x NuPAGE® transfer buffer (Invitrogen Ltd) (0.5M Bicine, 0.5 Bis-Tris, 20.5mM EDTA, 1mM Chlorobutanol) was diluted in 1x NuPAGE® transfer buffer containing 10% methanol, 1ml Antioxidant in final volume of 1L distilled water. Two sheets of Whatmann paper, a piece of nitrocellulose membrane (Hybond C^{extra}), and four blotting pads were soaked in transfer buffer and left until use. After electrophoresis, one of the gel cassette plates was removed and the wells were cut away. Two blotting pads were placed on the bottom plate (cathode) of the blotting apparatus, followed by a sheet of filter paper, then the gel, then the nitrocellulose membrane, then a sheet of filter paper and finally two blotting pads. Any trapped air bubbles were removed and the blotting was performed at constant voltage 30V for 80 minutes in 1x NuPAGE® transfer buffer containing 10% methanol. Transfer was checked by staining the nitrocellulose membrane with Ponceau S solution (Sigma).

2.2.47 Immunoblotting

Any non specific staining was avoided by blocking the membranes in 5% milk in phosphate buffer saline (PBS) containing 0.1 % Tween 20 for 1h at RT or overnight at 4°C. For detection of bovine MHC class I heavy chain, the membranes were incubated with 1:100 dilution of mAb IL-A88 antibody in 5% milk in PBS-0,1% Tween 20 for 2 hours at RT on shaker at lower speed. For detection of HLA-A2, the membrane was incubated with 1:1000 dilution of mAb HC-10 antibody in 5% milk in PBS-0,1% Tween 20. For detection of HLA-E, the membrane was incubated with 1:1000 dilution HLA-E antibody MEM-E/02 ($Abcam^R$) in PBS-0,1% Tween 20. For detection of mouse H_2d, the membrane was incubated with 1:100 dilution anti-H_2d antibody ($Cederlane^R$) in PBS-0,1% Tween 20. Protein

samples extracted from cyclohexamide and DMSO treated cell were incubated with mAb IL-A88 antibody at 1:100 in 5% milk in PBS-0,1% Tween 20. After 2x15 minutes wash in T-PBS for 15 minutes each, the membranes were incubated with 1:5000 dilution of sheep anti-mouse IgG hourseradish peroxidase linked whole antibody (Amersham Biosciences) in 5% milk in 1x T-PBS for 1hour at RT on an orbital shaker at lower speed. Following two washes as above, bound antibody was detected by incubating the membrane for 5 minutes at RT with ECL plus western blotting detection system (Amersham Biosciences). Any excess detection reagent was drained; the membrane was covered with a piece of saran wrap and placed in an x-ray film cassette (Hypercassette™ – Amersham Bioscences). In the dark room, a sheet of 18x24 X-ray film (Hyperfilm ™ Amersham Bioscences) was exposed to the membrane and developed in automatic x-ray film processor (Xograph IMAGING SYSTEMS- Compact X4). To carry out a control for these experiments, bound primary and secondary antibodies were removed from the membranes. The membranes were submerged in stripping buffer (100mM 2-Mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH6.7) and incubated at 50°C for 30 minutes with occasional shaking. After 2x10 minutes wash in T-PBS, the membranes were blocked in 5% milk in T-PBS for 1 hour at RT prior to re-incubating with 1:20000 dilution of anti-Actin antibody (Ab-1, CALBIOCHEM). Following 2 hours incubation with anti-actin Ab, the membranes were washed two times in T-PBS and incubated with anti-mouse IgG horseradish as described above.

Chapter Three

Results

3 Results

3.1 BPV-4 E5 protein down regulates MHC class I in bovine papillomas

3.1.1 Introduction

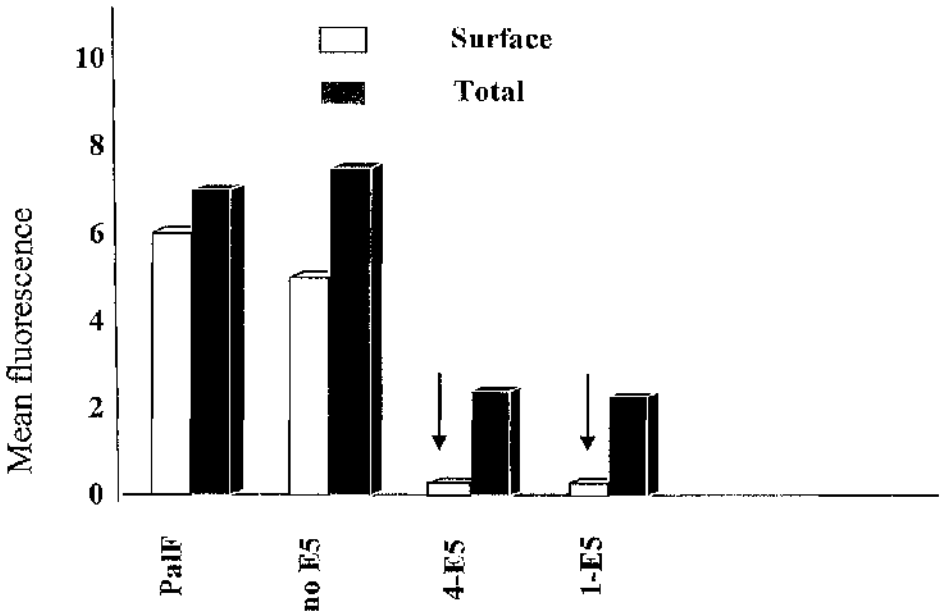
Infection by BPV-4 leads to the formation of proliferative lesions (warts) at the site of infection which start after four weeks of viral latency in the basal layer. The vast majority of papillomas regress through an activated T-cells immune response after a significant period of persistence which lasts for several months to one year. It has been suggested that persistence of papillomas is due to the inability of the immune system to recognize the viral antigens during the course of infection.

The analysis of BPV-4 DNA sequence revealed that it contains two transforming genes, E5 ORF and E7 ORF. The BPV-4 E5 ORF encodes a polypeptide only 42 amino acids long with a putative transmembrane domain comprising a highly hydrophopic N-terminal 30 residues and a hydrophilic C-terminal tail of 12 residues. It is mainly localized at the endosomal membranes, Golgi apparatus and occasionally the plasma membranes (Pennie et al 1993), and is expressed in the deep layers of early stages papillomas of the upper alimentary canal (Anderson et al 1997). E5 contributes to cell transformation by binding to ductin and inducing down-regulation of gap junction intercellular communication (GJIC) in transformed PalF cells (Faccini et al 1996). Furthermore, E5 increases cyclin A protein and cyclin A associated kinase activity, and inhibits the functions of the negative regulator of cell cycle $p27^{Kip1}$ (O'Brien and Campo 1998). Previous studies have shown that cells transformed by BPV-4 E5 show retention of MHC class I molecules in the Golgi apparatus and inhibition of transport of the complex to the cell surface (Ashrafi et al 2002; Marchetti et al 2002; O'Brien and Campo 2002). The MHC class I molecules play a fundamental role in the eradication of virally infected and transformed cells, since the cytotoxic T

cells can not recognize the infected cells unless foreign peptides are presented by MHC class I on the cell surface (Doherty and Zinkernagel 1975). The importance of MHC class I in virus clearance has led many viruses to establish numerous mechanisms of interference with the MHC class I pathway (Yewdell and Bennink 1999). The MIIC class I molecules are subdivided into two families, MHC class Ia or classical, and MIIC class Ib or non-classical. The classical MIIC class I molecules are polymorphic cell surface glycoproteins characterized by their ubiquitous expression in all nucleated cells. The lack of surface expression of these molecules as result of interference with their pathway hampers infected cell presentation of antigenic peptides to CD8 cells, leading to a decrease in immunological surveillance. It has been observed that expression of bovine papillomavirus E5 proteins in cultured bovine PalF cells induces dramatic reduction of surface expression of MHC I as well as it reduces the total amounts of MHC I to the half of amount compared with control cells (figure 4). The aim of this part of the study is to extend these observations to investigate whether BPV-4 E5 can down regulate MHC class I in natural infection.

Figure 4. BPV-4E5 downregulates MHC class I in bovine PalF cells. The mean forward fluorescence was calculated from the flow cytometry profiles of surface and total MHC class I in control (PalF, no E5 cells), and transformed cells expressing BPV-4E5 (Ashrafi et al., 2002).

Figure 4 BPV-4E5 down-regulates MHC class I in bovine PalF cells



From Ashrafi et al 2002

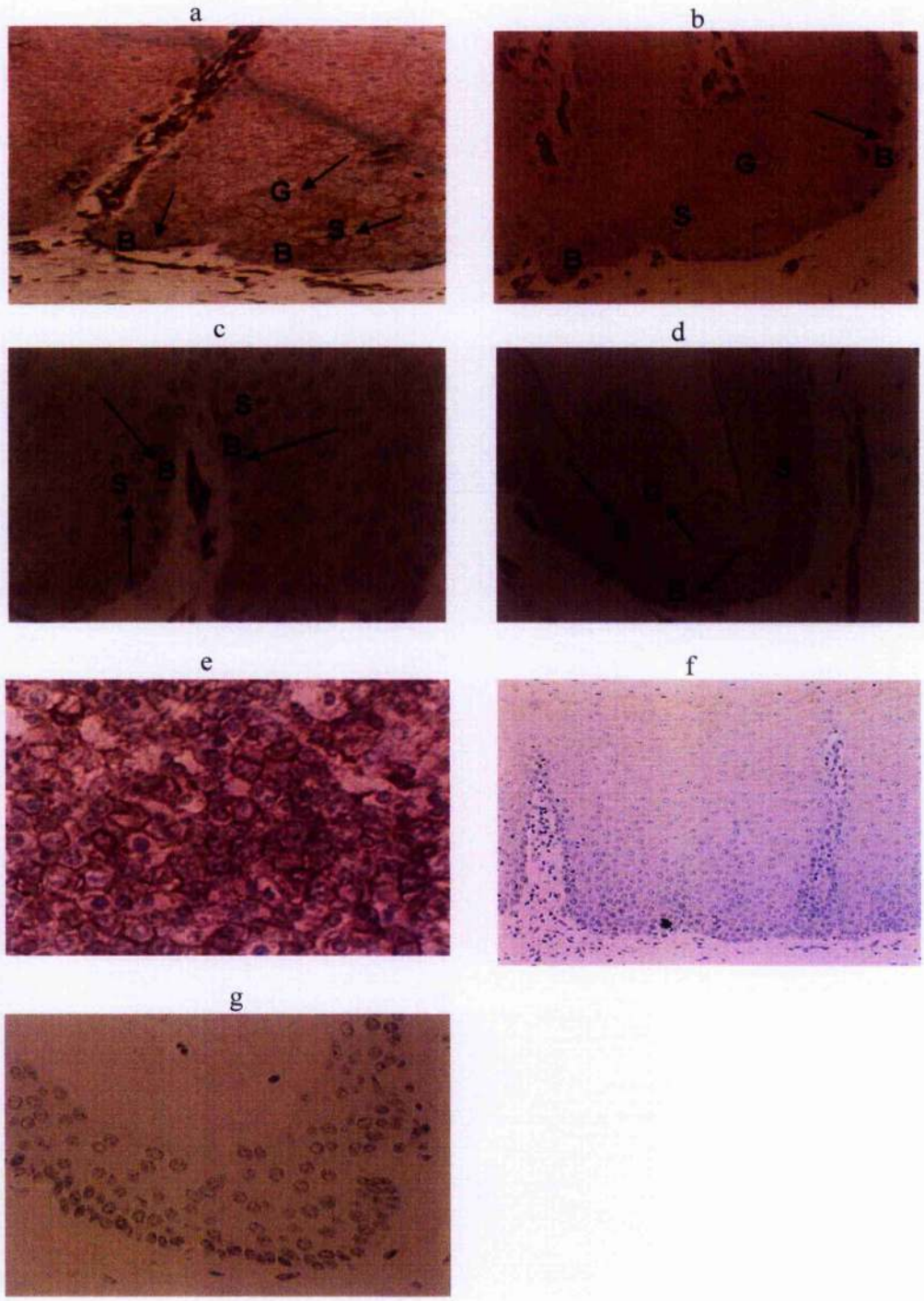
3.1.2 Expression of MHC class I in normal upper gastrointestinal tract

MHC class I genes encode glycoproteins expressed at the cell surface of almost all nucleated cells. To determine the feasibility of using the mAb IL-A88 to stain normal mucosa of bovine tissues and for evaluation the expression pattern of MHC I, sections from normal palate, tongue, buccal mucosa, and lymph node were deparaffinized and re-hydrated through a graded series of alcohol and endogenous peroxidase was inhibited in methanol containing 1.5% hydrogen peroxide. After antigen retrieval and blocking of sections with normal rabbit serum, sections were incubated with mAb IL-A88, which detects the heavy chain of bovine MHC I (Toye et al 1990). Sections were incubated with biotin-labeled rabbit anti-mouse secondary antibody. Signal was amplified by staining sections with avidin-biotin-peroxidase. MHC class I staining was visualised in brown colour by applying DAB to sections as described in section 2.2.9. In the epithelium, on the cell membranes, MHC I staining was observed with IL-A88 in almost all normal tissue specimens. There were slight differences in the pattern and the intensity of MHC I staining between the tissues; in normal buccal mucosa, the epithelium was stained throughout most of its thickness and staining on the surface of the cells was particularly clear in cells of the basal and suprabasal layers (figure 5-a). Although, all sections from tongue, hard palate, oesophagus, MHC I staining was mainly observed in the lower epithelial layers (figure 5-b,c and d respectively), some cells from basal and suprabasal layers did not show MHC I staining (figure 5-b,c). In contrast to epithelium, staining of lymph node with IL-A88 showed strong MHC I staining on the cell membrane of almost all T cells in a section (figure 5e). There was strong staining of the capillaries and stromal cells. The specificity of MHC I

staining was confirmed by incubating normal sections without mAb IL-A88 (figure 5-f,g).

Figure 5. Expression of MHC class I in paraffin sections from bovine normal samples stained with IL-A88. (a) buccal mucosa; MHC class I staining is observed on the cell surface throughout the whole thickness of epithelium from the basal layer (B), suprabasal (S) and granular layers (G) as indicated by arrows; (b,c) expression of MHC class I in tongue and hard palate respectively; some cells in basal (B) and suprabasal layers (S) do not express MHC class I as indicated by arrows; (d), MHC class I expression in oesophagus; there is clear cell surface staining in basal (B) and suprebasal layers (S) with no MHC staining in the upper layers (G) as indicated by arrows, (e), there is clear MHC class I expression on the cell surface of lymphocytes in a section of lymph node; (f,g), normal sections from buccal mucosa and oesophagus respectively were stained with secondary antibody only to verify the specificity of MHC class I staining. (a, b, d, e and g) x200 magnification, (c) x400 magnification, f x100 magnification.

Figure 5 Expression of MHC class I in normal upper gastrointestinal tract



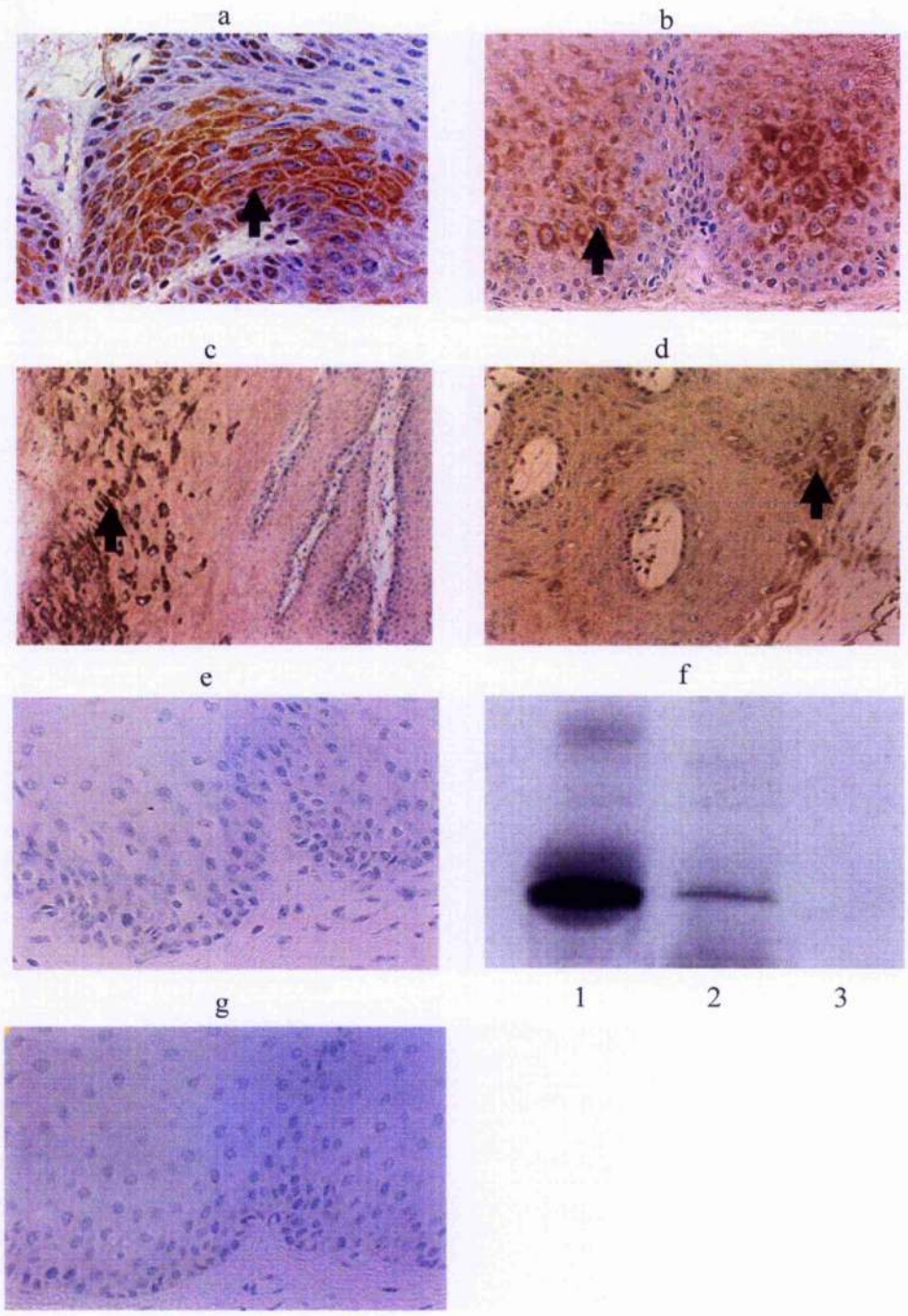
3.1.3 Expression of E5 in papillomas

To determine the expression of BPV-4E5 protein in natural infection, papilloma sections from the palate, rumen and oesophagus were deparaffinized and re-hydrated through a graded series of alcohol and endogenous peroxidase was inhibited in methanol containing 1.5% hydrogen peroxide. After blocking of sections with normal swine serum, sections were either incubated with 274 or 275 rabbit antiserum that was raised against a synthetic peptide representing the 12 C-terminal amino acids of the protein (Anderson et al 1997). Sections were incubated with biotin-labeled swine anti-rabbit secondary antibody. Signal was amplified by staining sections with avidin-biotin-peroxidase. E5 staining was visualised in brown colour by applying DAB to sections as described in section 2.2.9. E5 staining was observed exclusively in the cytoplasm of epithelial cells, from the basal and parabasal layers (figure 6-a,b) to the spinous and squamous layers (figure 6-c,d). Expression of E5 was, however, discontinuous; no papilloma was stained in all cell layers. The specificity of E5 staining was verified using several controls; pre-absorption of E5 antiserum was one of these controls, 10µl of E5 antiserum was incubated with 30µl (1mg/ml) of a synthetic E5 peptide representing the C-terminal fragment of BPV-4E5, against which the antiserum was raised, for 1 hour at 4°C. The complex of E5 peptide/ E5 antiserum bound was precipitated by centrifugation and the supernatant was used to investigate the elimination of E5 reactivity both *in vitro* and *in vivo* experiments. *In vivo*, the specificity of E5 staining was verified by incubation of papilloma section with preabsorbed E5 antiserum (figure 6-e). *In vitro* experiment was performed by B. Marchetti who showed that preabsorbed E5 antiserum failed to precipitate E5 (figure 6-f lane 3) compared with lane 2 in which E5 immunoprecipitated with E5 antiserum. In

addition, E5 reactivity was eliminated when serial section of papilloma 386 was incubated with secondary antibody only (figure 6-g).

Figure 6. Expression of BPV-4E5 in bovine papillomas. Tissue sections were immunostained for BPV-4E5 using two different E5 antisera (274, 275), raised in rabbit against E5 C-terminus domain. E5 expression was detected in cytoplasm of papilloma cells as indicated by arrowheads. E5 was mainly detected in basal and suprabasal layers using E5 antiserum 275 at dilution 1:5000 (a,b), and in the upper layers using E5 antiserum 274 at dilution 1:2000 (c,d). The specificity of E5 staining was verified by incubation of papilloma section with E5 antiserum preabsorbed with C-terminus peptide(e); (f); preabsorbed E5 antiserum failed to precipitate E5 (lane3); lane 1, E5 protein; lane 2, precipitated E5 protein by E5 antiserum. (g), staining of papilloma section with pre-immune serum. (a, b, d, e and g) x200 magnification, (c) x100 magnification.

Figure 6 Expression of BPV-4E5 in papillomas

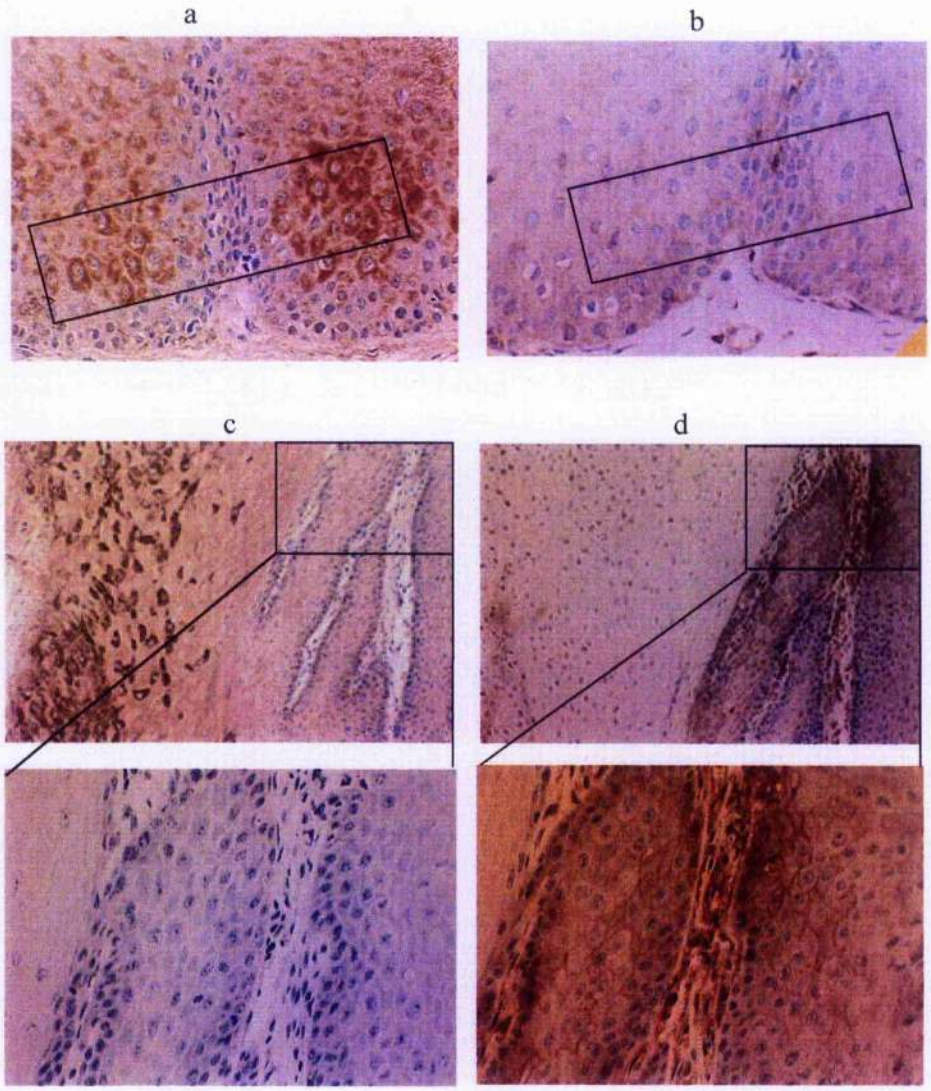


Expression of MHC class I in papillomas

To associate the expression of E5 with down regulation of MHC I, serial papillomas sections from the palate, rumen and oesophagus were either incubated with 274 or 275 E5 antiserum for detection of BPV-4 E5 or with mAb IL-A88 for detection of MHC I. Sections were incubated with biotin-labelled swine anti-rabbit secondary antibody for E5, while biotin-labelled rabbit anti-mouse was used for MHC I. Signal was amplified by staining sections with avidin-biotin-peroxidase. E5 or MHC I staining was visualised in brown colour by applying DAB to sections as described in section 2.2.9. Papilloma section stained with E5 antisera showed expression of E5 within the suprabasal and granular layers (figure 7a), however a serial section stained for MHC I with antisera IL-A88, revealed that the cells expressing E5 were negative for MHC I whereas cells without E5 expression demonstrated some staining for MHC I (figure 7b). MHC I was also strongly detected in stroma and capillaries. In all samples of papillomas, it is obvious that papilloma cells which did not show E5 staining (figure 7c), had detectable MHC I (figure 7d), and vice versa, cells lacking MHC I staining (figure 7d) had detectable cytoplasmic staining of E5 (figure 7c). Independently, of where cells expressing E5 localize within epithelial layers, expression of MHC I is not compatible with expression of E5. In agreement with previous work *in vitro* we conclude that expression of BPV-4 E5 in natural infection *in vivo* causes a remarkable reduction of MHC I expression on the surface of papilloma cells, thus potentially allowing virus to escape immune recognition.

Figure 7. Expression of MHC class I in papillomas. Serial sections of papillomas were stained with either mAb IL-A88 or with E5 antiserum. Slides were microscopically examined by looking at E5 staining patterns in particular areas and matching them with the same areas in the section stained for MHC class I. Images were captured using computerized images analyser (Zeiss KS300) at x 200 magnification. Papilloma cells that expressed E5 (a) did not express MIIC I (b) as indicated by rectangles. Vice versa, papilloma cells which did not express E5 (c) had detectable MIIC class I (d) as indicated by enlarged squares. (a and b) and the bottom of c and d x200 magnification, the top of c and d x100 magnification.

Figure 7 Expression of MHC class I in papillomas

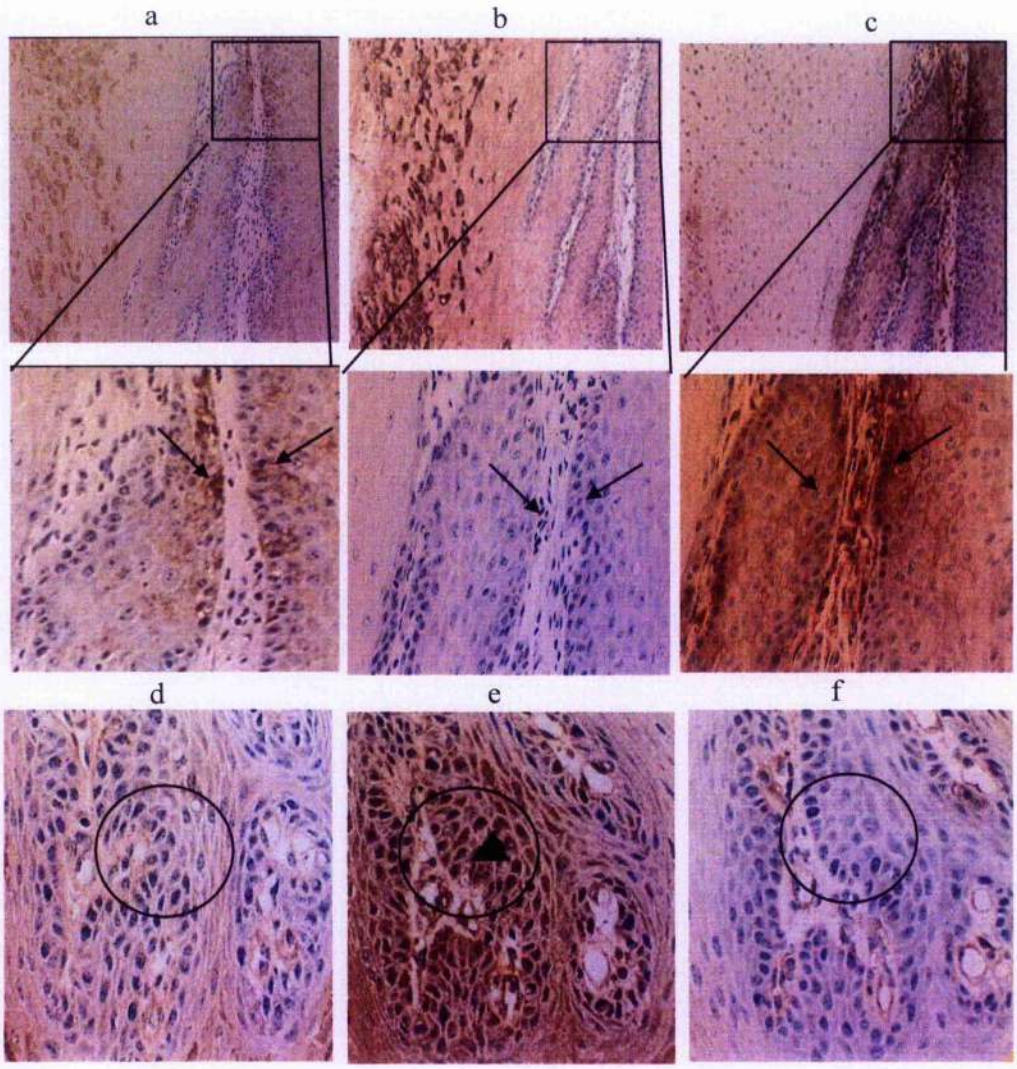


Down-regulation of MHC class I is due to expression of E5 but not of E7

It has been reported that E7 proteins of HPV-16 and -11 are implicated, respectively, in the down-regulation of MHC I either through inhibition of the transcriptional promoter of the MHC I heavy chain (Georgopoulos et al 2000) or indirectly through inhibition of TAP, the transporter associated with peptide (Vambutas et al 2001). To ensure that the absence of MHC I in bovine papillomas was due to E5 and not to E7, serial papillomas sections were either incubated with mAb IL-A88 for detection of MHC I, with E5 antiserum for detection of BPV-4 E5 or with rabbit antisera 11547 and 11823, which were raised against a β-galactosidase–E7 fusion protein (Anderson et al 1997) for detection of BPV-4 E7. Sections were incubated with biotin-labeled swine anti-rabbit secondary antibody for labelling E5 Ab/Ag bound and E7 Ab/Ag bound, while biotin-labeled rabbit anti-mouse was used to label mAb L-A88/ MHC I bound. Signal was amplified by staining sections with avidin-biotin-peroxidase. E5, MHC I and E7 staining were visualised in brown colour by applying DAB to sections as described in section 2.2.9. Cells expressing either E5 or E7 alone were seldom detected because E5 ORF is located between the long control region and the E7 ORF and both E5 and E7 are co-expressed in the initial stages of the infection. Papilloma cells that express E7 (figure 8a), but not E5 (figure 8b), still had detectable MHC I (figure 8c). Conversely, cells that did not express E7 (figure 8d) but expressed E5 (figure 8e), there was little or no MHC I (figure 8f). These results confirm that expression of BPV-4E7, in contrast to HPV E7 is not responsible for down-regulation of MHC I and confirmed the previous observations that E5 is only the viral protein of BPV-4 responsible for down-regulation of MHC I.

Figure 8. Expression of BPV-4E7 does not interfere with expression of MHC class I. Serial sections of papillomas were either incubated with mAb IL-A88 for detection of MHC I, with E5 antiserum for detection of BPV-4 E5 or with rabbit antisera 11547 and 11823, which were raised against a β-galactosidase–E7 fusion protein. Slides were microscopically examined by looking at E5 staining patterns in particular areas and matching them with the same areas in the section stained for MHC class I. Images were captured using computerized images analyser (Zeiss KS300) at x 200 magnification. Papilloma cells that expressed E7 (a), but not E5 (b) still had detectable MHC class I (c) as indicated by arrows in enlarged areas. Papilloma cells that did not express E7 (d) but expressed E5 (e) had no MHC class I (f) as indicated by circles. (a, b and c) x100 magnification, (d, e and f) x200 magnification.

Figure 8 Co-expression of MHC class I and BPV-4E7 in bovine papillomas



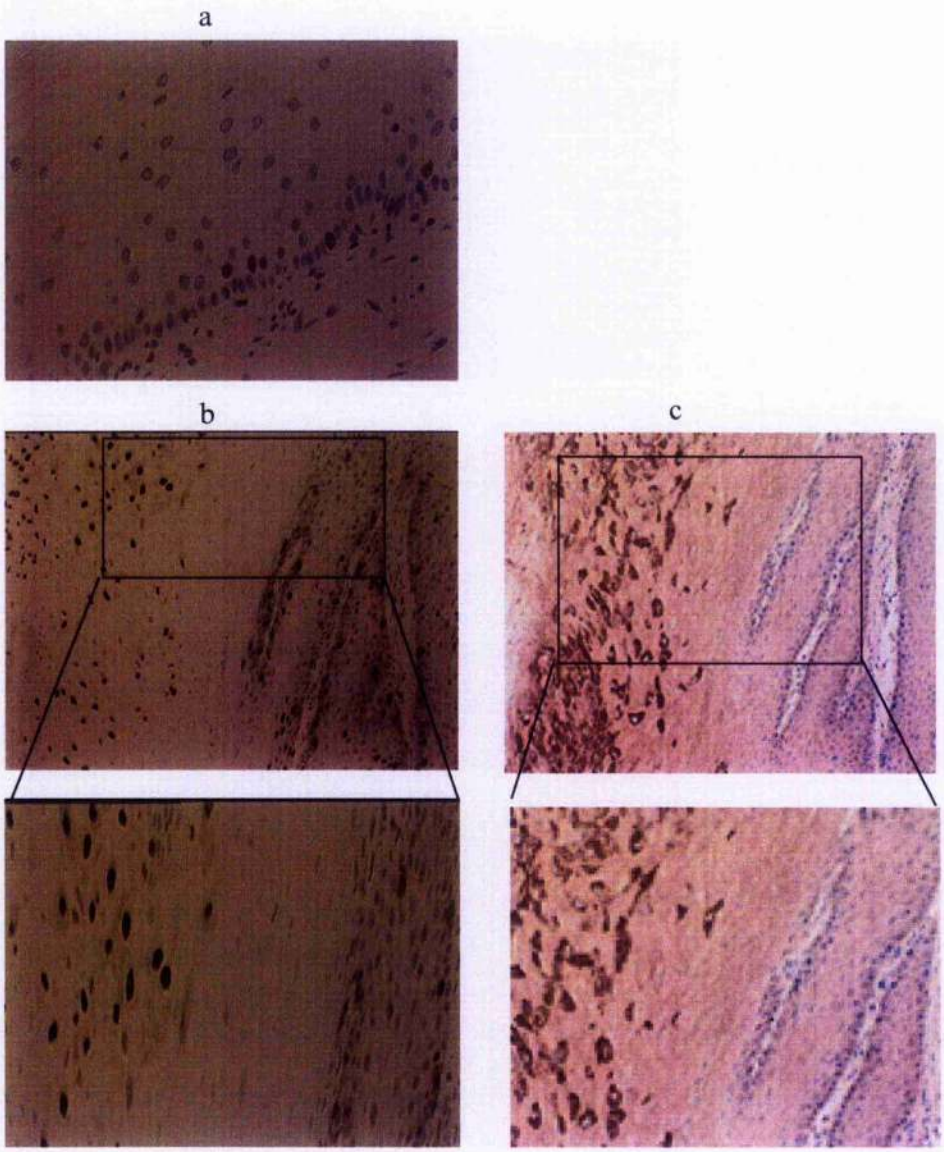
3.1.6 Evaluation of the cell proliferative state in papillomas

In vitro, E5 has been shown to have several biological activities which manipulate cell functions and lead to cell transformation. To evaluate the proliferative state of papilloma cells, proliferation marker Ki67 expression was used to assess the relationship between E5 expression and cellular proliferation. The Ki-67 is a proliferation antigen which is expressed during all phases of the cell cycle. However, Ki67 antigen is consistently absent in quiescent cells (G0) and is not detectable during DNA repair processes (Hall et al 1993). The Ki67 antigen is predominantly localized in the perinuclear region and has an important role in the maintenance and regulation of the cell division. Ki-67 is currently considered to be the best proliferative marker for clinical use because of its reliability and simplicity (Brown and Gatter 1990; Mengel et al 2002). Serial sections of normal tissues and papillomas were either incubated with E5 antiserum or with mAb MIB-1 for detection of expression of the proliferation antigen Ki67 as described in section 2.2.9. As expected, in normal epithelium, expression of Ki67 was restricted to basal and suprabasal cells and was absent in differentiated cells (figure 9a) because these cells once differentiated are no longer permitted to proliferate. In contrast, in papilloma, expression of Ki67 was detected in the basal cells and in E5-expressing differentiated keratinocytes (figure 9b). Except from basal cells that did not show E5 expression, expression of Ki67 was remarkably associated with expression of E5 (figure 9c). Although, sequences analysis has revealed that BPV-4E7 shares homology with HPV-16E7, it contains an LXCXE motif that binds the Rb protein thus contributing to cell cycle progression. However, it is not proved yet whether BPV-4E7 contributes to cell cycle progression by interfering with Rb function. In contrast, based on *in vitro* data, BPV-4E5 has been shown to induce proliferation

of cells maintained in suspension and in low serum and cellular growth was correlated with an increase in cyclin A and cdk expression (O'Brien and Campo 1998). For BPV-4, expression of E5 has been shown in previous literature (O'Brien and Campo 1998) to be more effective on the cell cycle progression than BPV-4E7. It should be noted however, that although BPV-4E7 possess the relevant motif associated with binding of Rb, this has not been formally proven. In addition, no correlation was made between BPV-4 E7 and Ki67 expression in papillomas. These results would suggest that expression of BPV-4 E5 in papillomas delays cellular differentiation by interfering with normal regulators of cell cycle and induces post-mitotic cells to re-inter S-phase and establish proliferation.

Figure 9. Expression of the proliferation marker Ki67 in papillomas. Representative papilloma sections were either incubated with mAb MIB-1 at dilution 1:200 for detection of Ki67 or with E5 antiserum at dilution 1:2000 for detection of BPV-4 E5. Normal sections from gastrointestinal tract were also stained with mAb MIB-1. Slides were microscopically examined by looking at E5 staining patterns in particular areas and matching them with the same areas in the section stained for Ki67. Images were captured using computerized images analyser (Zeiss KS300) at x100 and x 200 magnification. a) In normal section, only a few cells in the basal layer were positive for Ki67. b) In papillomas, in addition to its detection in the basal cells, Ki67 was detected in the cells of the upper layers which had detectable BPV-4E5 (c) as indicated by rectangles and enlarged areas. (a and the bottom of b and c) x200 magnification, the top of b and c x100 magnification.

Figure 9 Co-expression of Ki67 and BPV-4E5 in bovine papillomas



3.2 BPV-4 E5 protein does not down regulate bovine non classical MHC I

3.2.1 Introduction

BPV-4 E5 protein causes a significant reduction in the expression of MHC class I molecules on the cell surface. Cells that express no or low levels of MHC class I on the cell surface are subjected to natural killer NK cell lysis. It has been shown that human non classical molecules of MHC class I (HLA-E) is a major legend for the CD94-NKG2 receptors expressed by natural killer (NK) cells. Interaction between HLA-E and NK receptors blocks the action of the activation receptors and inhibits the NK cells from being activated. Therefore, the presence of non classical molecules on the cell surface protects cells from NK cytotoxicity. The non-classical MHC class I molecules show homology to classical class I molecules but generally have limited polymorphism, low cell surface expression, and more restricted tissue distribution (Shawar et al 1994). It has been shown that several viruses are capable of inhibiting classical MHC I but do not inhibit non-classical MHC I thus escaping both CTLs and NK cells. It has been demonstrated that the human immunodeficiency virus (HIV) Nef and human cytomegalovirus (HCMV) US10/UL40 proteins down-regulate HLA-A/B but do not down-regulate HLA-C/E, which are mostly involved in positive selection of inhibitory receptors on NK cells (Braud et al 2002; Wang et al 2002).

3.2.2 Generation of mouse P815 cells expressing bovine MHC class I

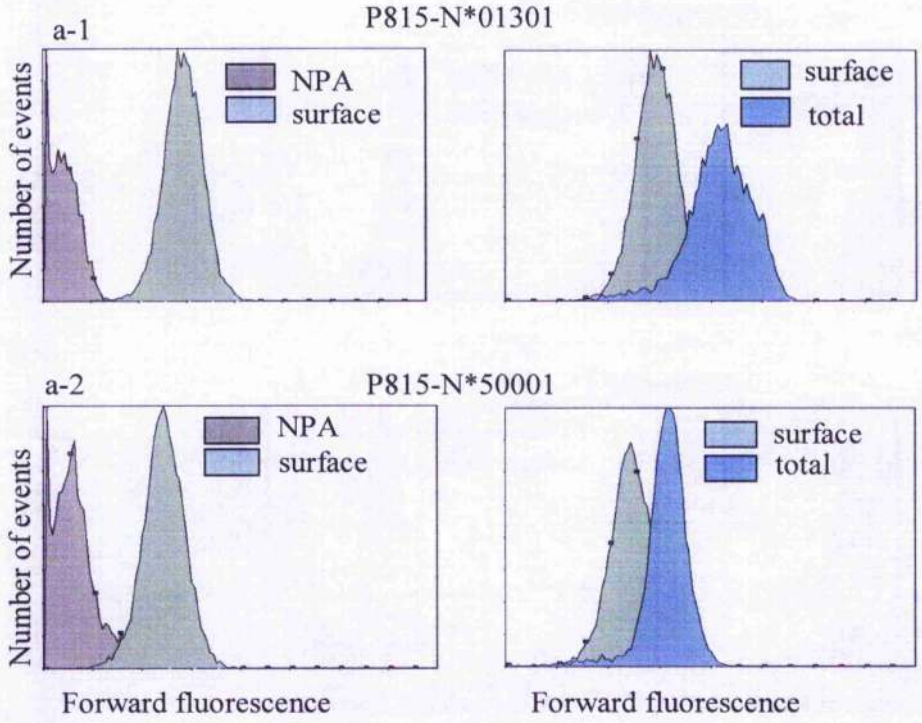
Unlike human non classical HLA-E which, can be detected by specific antibody, non classical MHC class I can not be detected in bovine cells expressing classical molecules, because the available antibody IL-A88 cross reacts with all bovine

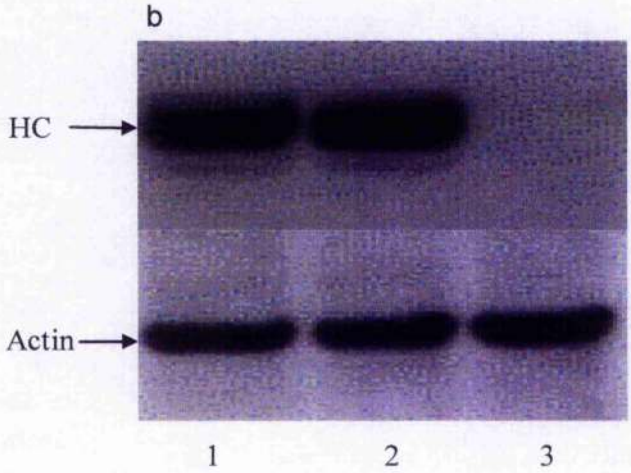
MHC class I heavy chains. To determine whether BPV-4 E5, like HPV-16 E5 (Ashrafi et al 2005), is incapable of down-regulating certain non-classical MHC genes, we used a mouse mastocytoma P815 cell line either transfected with bovine classical (pcDNA6-V5-His-*N*01301)*, or bovine non classical (pcDNA6-V5-His-N*50001*)* MHC class I.

*N*01301* is one of bovine classical MHC I genes originally termed HD6. *N*01301* belongs to the A18 haplotype, which is one of the extensively characterized haplotypes (Ellis et al 1996). *N*50001* is a bovine non-classical class I gene, first identified in 1995 when it was termed *Gene X* and more recently termed HD59 (Ellis et al., 1996). *N*50001* and other putative bovine non-classical class I gene HD15, are not closely related to any of the bovine classical class I genes (Holmes et al 2003). *N*50001* encodes a HC with a truncated cytoplasmic domain (8 rather than 28 amino acids), and in this respect resembles human HLA-G (Geraghty et al 1987). The *N*50001* cDNA and *N*01301* cDNA were introduced into the mouse cell line P815, giving rise to P815-N*50001 cells and P815-N*01301 cells. There was no need to co-transfect the bovine β_2 microglobulin (β_2m) gene with the bovine MHC HC genes into P815 cells because the bovine class I HC associates with mouse β_2m, and when the complex reaches the cell surface it rapidly exchanges mouse β_2m with bovine β_2m provided by the foetal calf serum in the culture medium (Ellis et al., 2005). Transfected P815 cells were selected in medium containing 10μg/ml Blasticidin for 21 days. Positively selected cells were screened for HC expression by FACS analysis (figure 10a) and western blot (figure 10b) with mAb IL-A88. Both sets of results showed that bovine classical and non classical MHC I are expressed in P815 cells. No cross reaction of mAb IL-A88 with mouse MHC I was observed (figure 10b lane 3)

Figure 10. Expression of N*01301 and N*50001 in P815 cells. Parental P815 cells were either transfected with bovine classical N*01301 or non classical N*50001 MHC class I, selected in medium containing 10μg/ml Blasticidin for 21 days and positively selected cells were stained with mAb IL-A88 for MHC I expression. (a-1) FACS analysis histograms representing both surface and total expression of N*01301. (a-2) FACS analysis histograms representing both surface and total expression of N*50001. In a-1 and a-2, NPA represents N*01301 and N*50001 cells stained with secondary antibody only. (b) immunoblotting of N*01301 (lane 1) and N*50001(lane 2) heavy chains, no cross reaction of mAb IL-A88 with mouse MHC I was observed (lane 3).

Figure 10 Expression of bovine MHC class I in P815 cells





3.2.3 BPV-4 E5 constructs

BPV-4 E5 ORF was available into two different vectors: pcDNA and pZipneo giving rise to pc4E5 and pZip4E5 respectively (obtained from Dr Ashrafi, University of Glasgow). pcDNA is a universal expression vector in which the inserted gene is under the control of the CMV early promoter, pZipneo is a plasmid in which expression is directed by MoMuLV long terminal repeat LTR promoter. The sequence coding for the influenza virus haemaagglutinin type-1 (HA1) epitope was inserted at the 5' of the E5 sequences.

3.2.4 Transfection of BPV-4 E5 in P815 cells

Mouse mastocytoma P815 cell lines (control and expressing bovine HC) were stably transfected with a range of plasmid DNA, pcDNA, pc4E5, pZipneo, and pZip4E5, by using either transfection reagents (GeneJuice) or the Nucleofector apparatus (Amaxa- Germany), to investigate the role of BPV-4 E5 on MHC class I. One day prior to transfection, 1x 10^6 P815 cells were placed in 6-well plates with appropriate medium and incubated at 37°C in 5% CO_2 overnight following the transfection protocol as described in section 2.2.18. For introduction of BPV-4 E5 by Nucleofector, 1x 10^6 cells were pelleted and resuspended in 100 µl transfection reagent (Mouse cells transfection kit). After adding 4µg of the plasmid DNA to the cell mixture, cells were applied to Nucleofector following the transfection protocol. Following transfection, the cells were selected in medium containing 500µg/ml G418 for 21 days.

3.2.5 Expression of BPV E5 in P815 cells

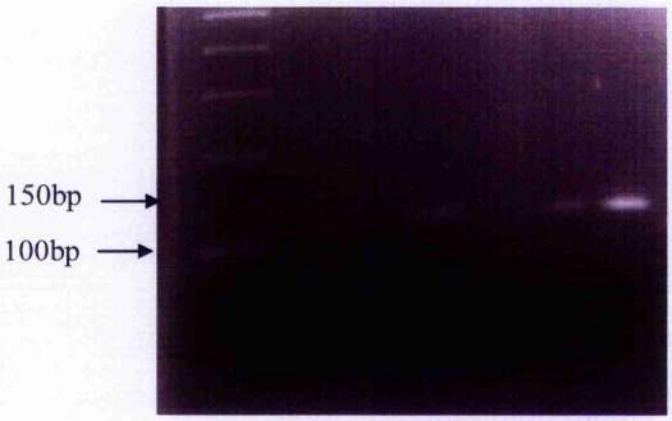
BPV-4 E5 is a very small hydrophobic protein, only 42 amino acids long and it is expressed at low levels in cells (Anderson et al 1997). Introduction of pZip4E5 into P815 cells by using Gene Juice did not give satisfactory results and cells did not survive selection even after co-transfection with pZip plasmid carrying pT24 activated *ras* gene and pZipBPV-4 E7 (Jaggar et al 1990; Pennie et al 1993). When the E5 plasmid was changed to pc4E5 instead of pZip4E5, transfectants could be selected. E5 DNA was detected by PCR (figure 11a) using the following primers: 4E5 Forward 5' CCA TAC GAT GTT CCA GAT TAC GCT 3', 4E5 Reverse 5' CCA TCC ATC TAA CCG AGT AAT AGT 3'. However, despite the presence of the E5 ORF, there was no E5 expression when examined by quantitative RT-PCR, although actin RNA was easily detected (figure 11b). Nucleofector is very useful tool for introducing genes of interest in many cell lines; this could be due to the production of high electrical pulses that help introducing the gene into the cell genome. By using Nucleofector, many more E5 transfectants were isolated from these cells. RNA was isolated, purified from any residual DNA and typically 1μg of purified RNA from each sample was used for quantitative RT-PCR. We analysed two pc4E5 and two pZip4E5 pools expressing *N*01301* or *N*50001* for the presence of E5 transcripts (figure 12a 1,2,3). In each experiment, additional reactions, with 10 fold serial dilutions of template DNA, were performed with each set of primers and probes on the same 96-well plates to generate standard curves. Quantitive RT-PCR was used to determine the relative amount of BPV-4 E5 and mouse β-actin mRNA in triplicate experiments for each E5 transfected cell line and cells carrying empty vectors by using a standard curve (figure 12b). The results show that all of the transfected cells (pc4E5 and pzip4E5) express the E5 gene but

at 1,000 fold less than β-actin. As expected, no E5 RNA was detected in the control cells. This low level of E5 expression is in agreement with previous results *in virto* consistently obtained in all BPV-4 E5-transfected NIH 3T3 and bovine PalF cell lines (Ashrafi et al 2002; Marchetti et al 2002).

Figure 11. Detection of BPV-4E5 by PCR. DNA samples from P815 cells transfected with BPV-4E5 by using GeneJuice were extracted and amplified using appropriate primers for detection of BPV-4E5 DNA. E5 fragment was detected in samples extracted from cells expressing N*01301 and N*50001. 11a; lane 1 DNA marker; lane 2, no template; lane 3, N*01301pcDNA; lane 4, N*01301pc-4E5; lane 5, N*50001pcDNA; lane 6, N*50001pc-4E5; lane 7, 4E5 plasmid. Quantitative RT-PCR shows no E5 expression in P815 cells in which E5 ORF was detected, although actin RNA was easily detected (b).

Figure 11 Detection of BPV-4E5 DNA but not E5 mRNA in P815 cells

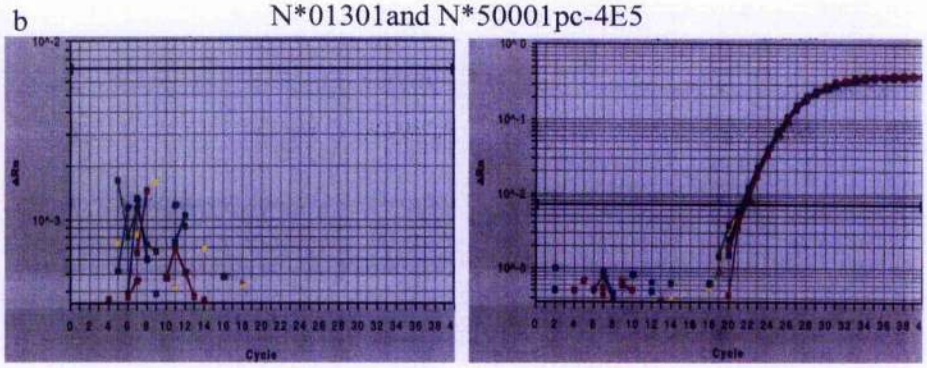
a



1 2 3 4 5 6 7

1- DNA Marker
2- No template
3- N*01301pcDNA
4- N*01301pc-4E5
5- N*50001pcDNA
6- N*50001pc-4E5
7- 4E5 plasmid

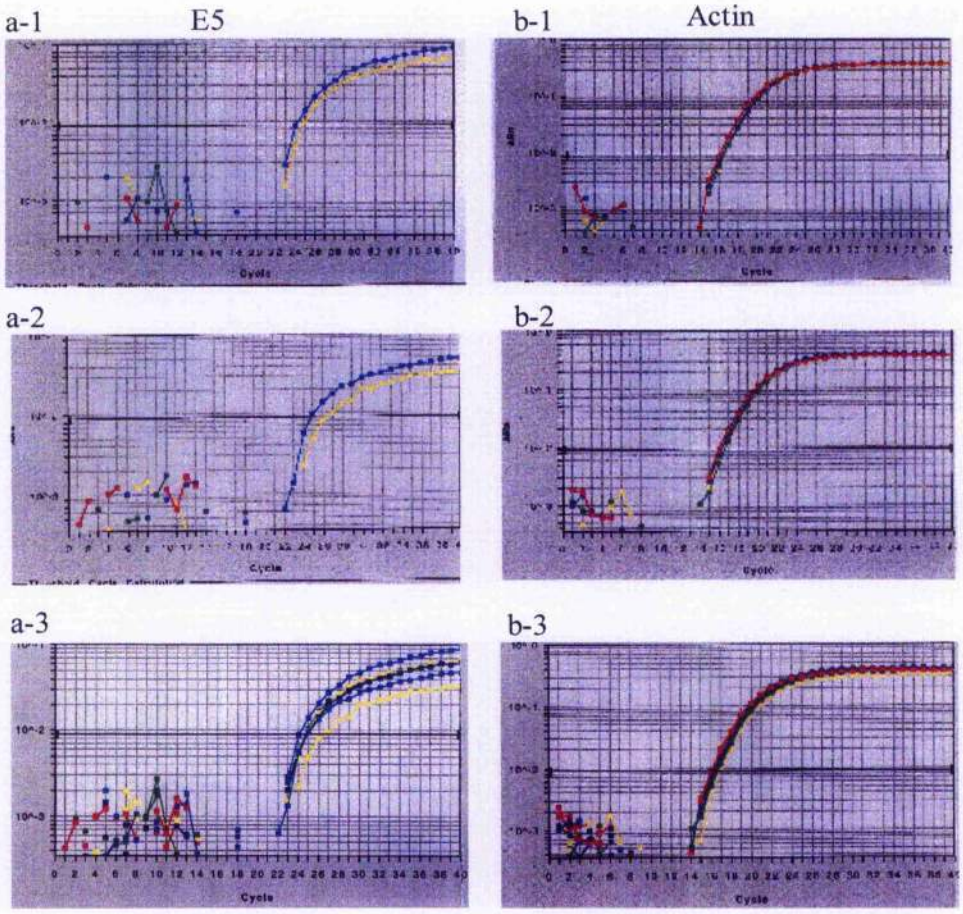
b



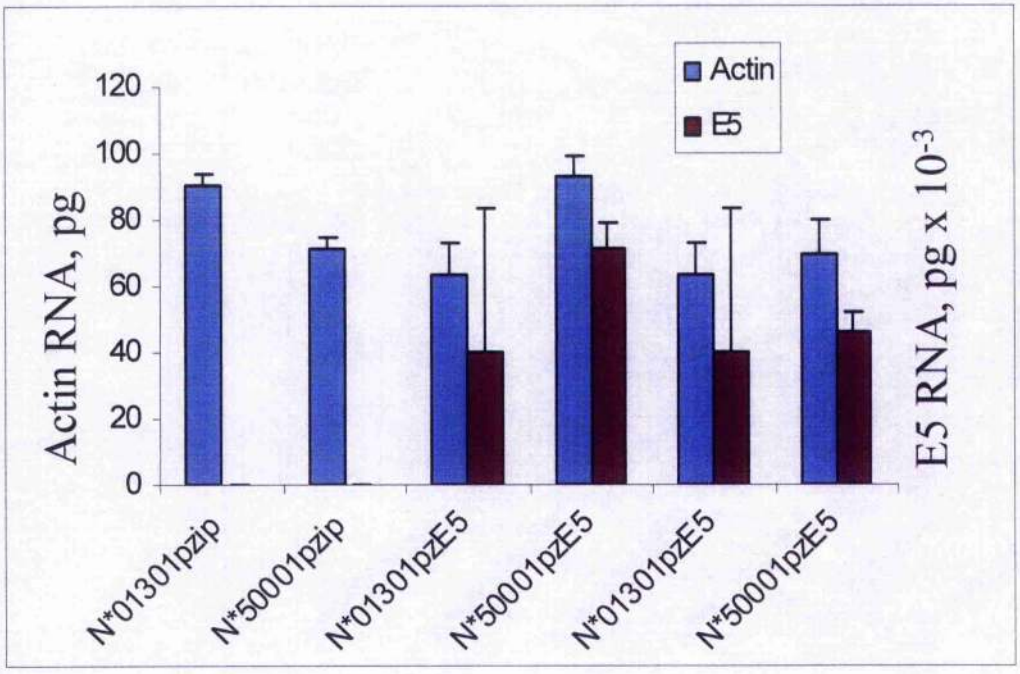
E5 Actin

Figure 12. Taq-Man RT-PCR was used to determine the expression of BPV-4E5 mRNA in P815 cells transfected by using Nucleofector. (a) The expression of BPV-4 E5 RNA and actin mRNA was determined in P815 control cells (pcDNA and pZip) or cells expressing BPV-4E5 (two pc4E5 and two pZip4E5 pools expressing N*01301 or N*50001), a-1 represents expression of E5 mRNA in P815-N*01301 cells, a-2 represents expression of E5 mRNA in P815-N*50001 cells, while a-3 represents expression of E5 mRNA both in P815-N*01301 and P815-N*50001 cells. The histograms represent the average with standard deviation of six independent measurements. (b) Note that the levels of E5 RNA are three orders of magnitude lower than those of β-actin RNA.

Figure 12 Expression of BPV-4E5 mRNA in transfected P815 cells



b

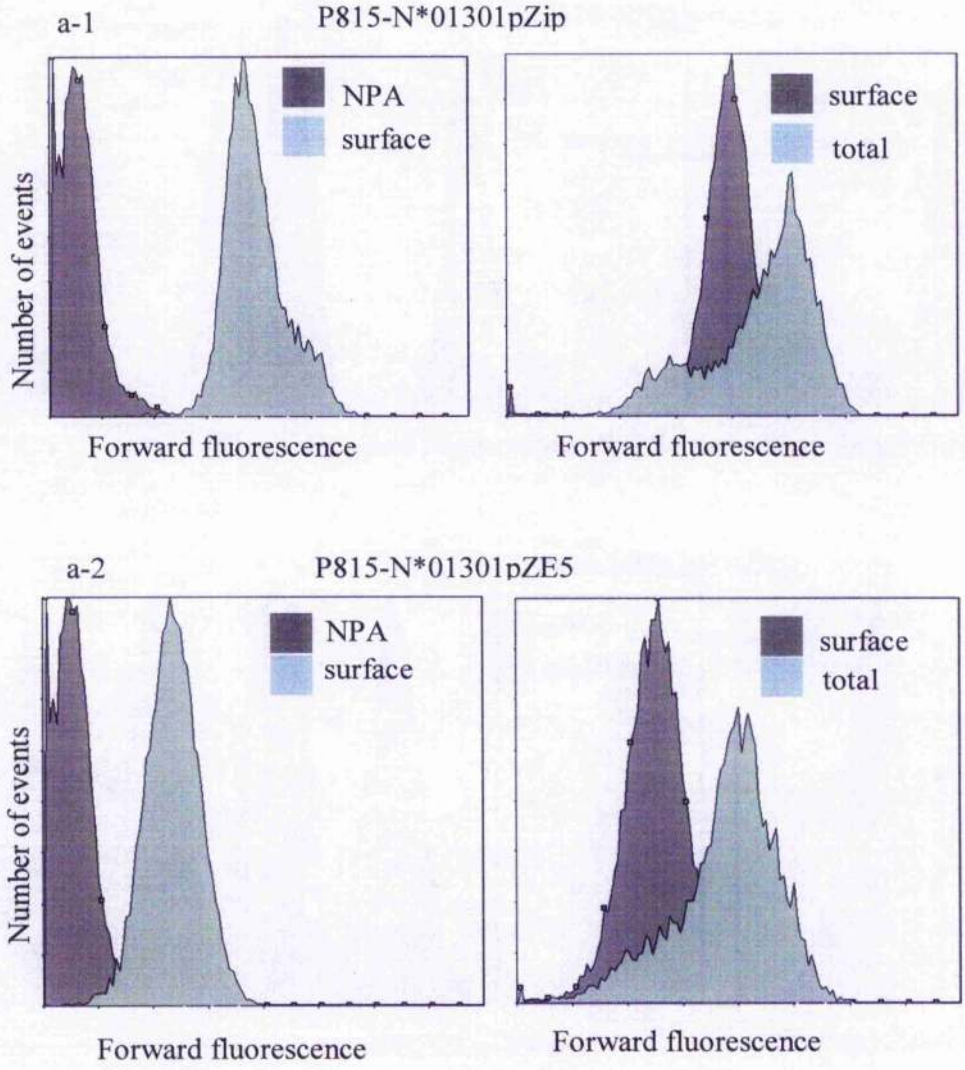

Actin
E5
120
100
80
60
40
20
0
Actin RNA, pg
E5 RNA, pg x 10-3
N*01301pzip
N*50001pzip
N*01301pzE5
N*50001pzE5
N*01301pzE5
N*50001pzE5

3.2.6 Expression of bovine classical MHC class I in P815 cells expressing BPV-4E5

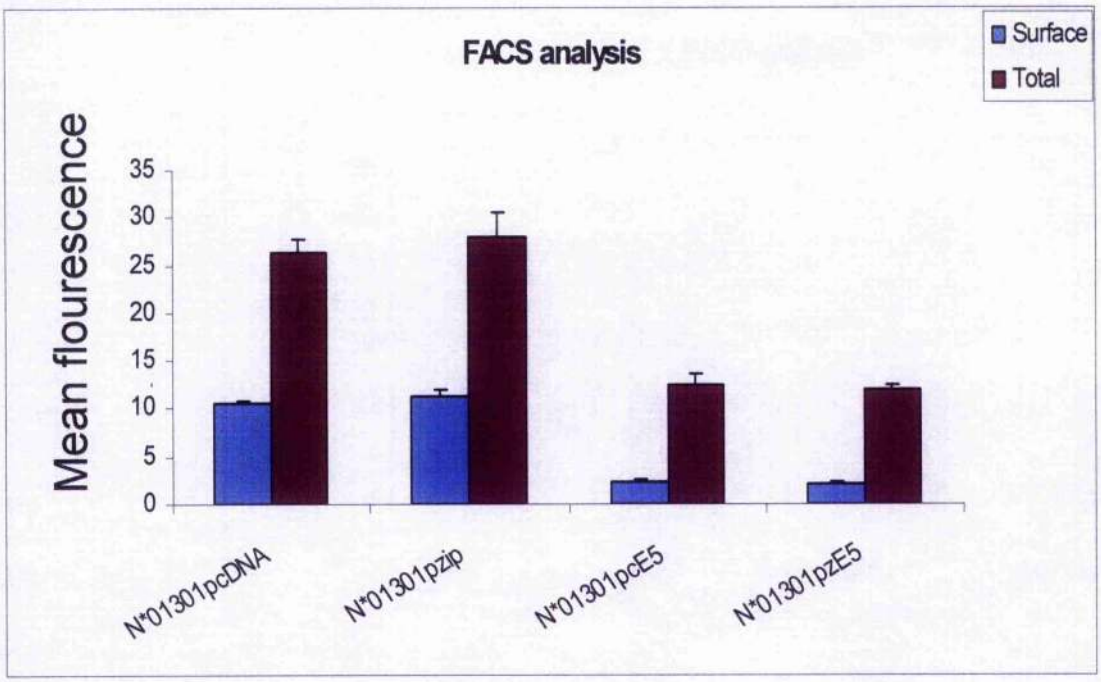
To determine the expression of classical N*01301 in E5 cells, flow cytometry experiments were performed as described in section 2.2.40. P815-N*01301 cells harbouring empty vector or expressing BPV-4E5 were incubated with mAb IL-A88, and analysed for levels of both surface and total N*01301. The results show that in cells carrying pZip empty vector, there was a remarkable shift in fluorescence for both surface and total of N*01301 when compared with cells stained with only FITC conjugated secondary antibody (figure 13a-1). In contrast, in cells expressing BPV-4E5, there was a remarkable reduction both in surface and total expression of N*01301 as indicated by a reduction of fluorescent shift when compared with control cells (figure 13a-2). To quantify the level of surface and total of N*01301, at least four experiments were performed and the mean of forward fluorescence shift was calculated. There was very little surface MHC class I, with a reduction of more than five fold compared to control cells carrying empty vector, and also the amount of total MHC class I was less than half of that in control cells (figure 13b). There was no difference between results obtained with P815-N*01301-pc4E5 cells and with P815-N*01301-pz4E5. The results confirm that 4E5 is capable of down-regulating MHC class I in agreement with previous work *in vitro* (Ashrafi et al 2002; Marchetti et al 2002). Also the results show that BPV-4E5 is capable of down-regulating MHC class I in the absence of any other viral oncogene.

Figure 13. BPV-4E5 down-regulates surface and total classical N*01301 MHC class I. a-1) Example of flow cytometry profiles of surface and total classical N*01301 MHC class I in P815 control cells (pZip) stained with mAb IL-A88. a-2) Example of flow cytometry profiles from P815-N*01301 cells expressing E5 (pZ-4E5) stained with mAb IL-A88. NPA, no fluorescence shift from N*01301pZip or N*01301pZ-4E5 cells stained with FITC conjugated secondary antibody only (a-1 and a-2). b) Mean forward fluorescence with standard deviation calculated from the flow cytometry profiles of surface and total classical N*01301 MHC class I in P815 control cells (pcDNA and pZip) or cells expressing E5 (pc-4E5 and pZ-4E5) of at least four independent experiments in duplicate.

Figure 13 BPV-4E5 down-regulates expression of surface and total N*01301



b

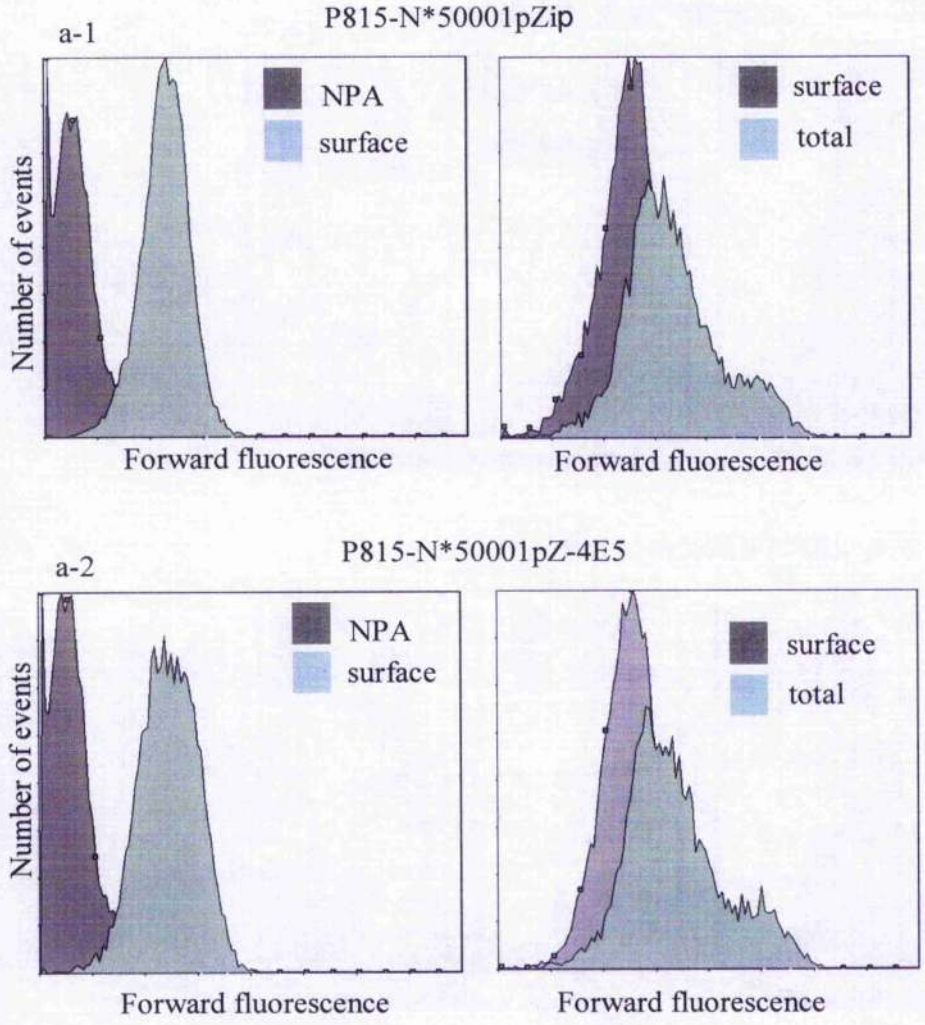

FACS analysis
Surface
Total
Mean flourescence
35
30
25
20
15
10
5
0
N°01301pcDNA
N°01301pzip
N°01301pcE5
N°01301pzE5

3.2.7 Expression of bovine non-classical MHC class I in P815 cells expressing BPV-4E5

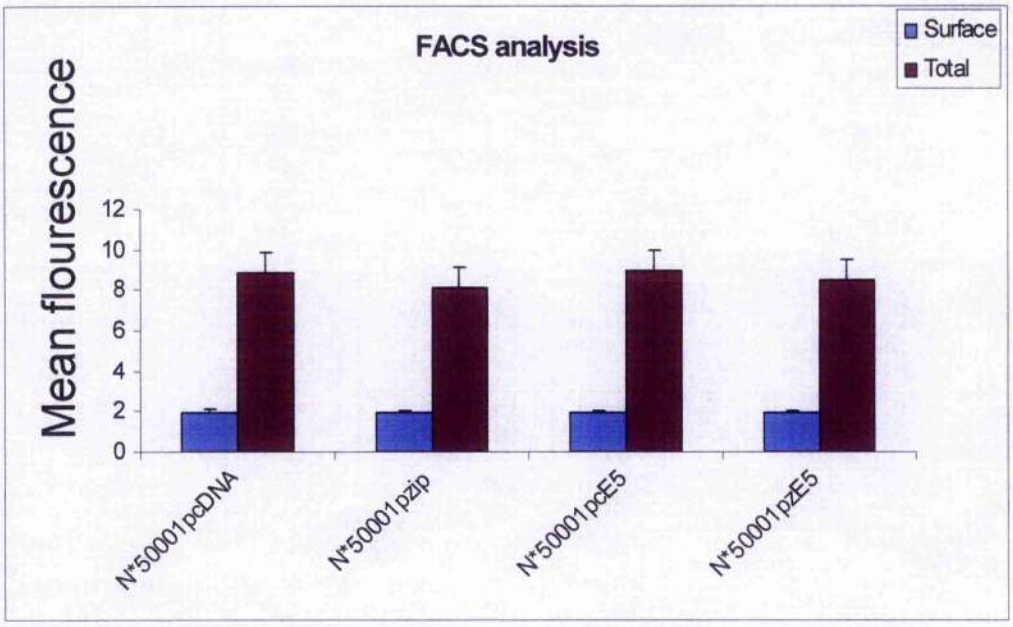
To determine the expression of non classical P815-N*50001 in E5 cells, flow cytometry experiments were performed as described in section 2.2.40. P815-N*50001 cells harbouring empty vector or expressing BPV-4E5 were incubated mAb IL-A88, analysed for levels of both surface and total N*50001. The results showed there was no significant difference either in surface or total expression of N*50001 between empty vector and E5 cells, indicated by the similarity in fluorescence shift between empty vector and E5 cells (figure 14a-1,2 respectively). To quantify the level of surface and total non classical MHC I in E5 cells, at least four experiments were performed and the mean of forward fluorescence shift was calculated. The results showed that expression of N*50001 was not at all affected by E5 and the levels of surface or total MHC I were indistinguishable from those in control cells (figure 14b). There was no difference between results obtained with P815-N*50001-pc4E5 cells and with P815-N*50001-pZ4E5. The results show that BPV-4E5 is incapable of down regulating non-classical MHC I.

Figure 14. BPV-4E5 does not down-regulate surface and total non classical N*50001 MHC class I. a-1) Example of flow cytometry profiles of surface and total non classical N*50001 MHC class I in P815 control cells (pZip) stained with mAb IL-A88. a-2) Example of flow cytometry profiles of surface and total non classical N*50001 MHC class I in P815 cells expressing E5 (pZ-4E5) stained with mAb IL-A88. NPA, no fluorescence shift from N*50001pZip or N*50001pZ-4E5 cells stained with FITC conjugated secondary antibody only (a-1 and a-2). b) Mean forward fluorescence with standard deviation calculated from the flow cytometry profiles of surface and total non classical N*50001 MHC class I in P815 control cells (pcDNA and pZip) or cells expressing E5 (pc-4E5 and pZ-4E5) of at least four independent experiments in duplicate.

Figure 14 BPV-4E5 does not down-regulate N*50001



b


FACS analysis
Surface
Total
Mean flourescence
12
10
8
6
4
2
0
N*50001pcDNA
N*50001pzip
N*50001pcE5
N*50001pzE5

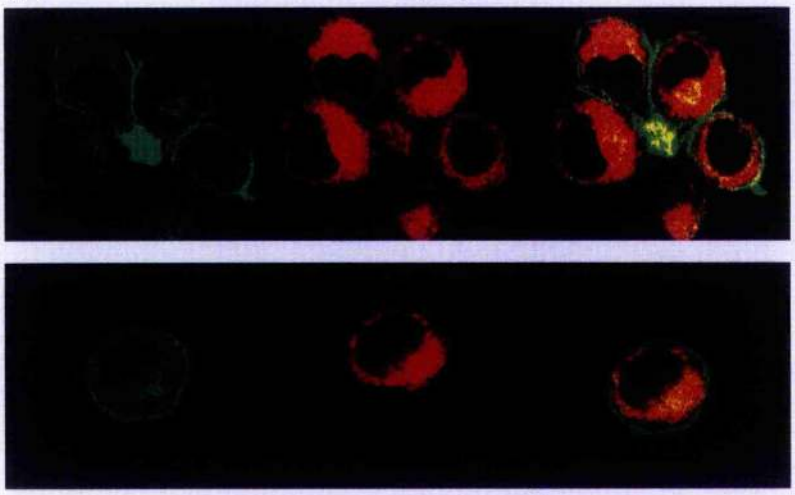
3.2.8 Localization of classical MHC class I in P815 cells

In PalF-4E5 cells, E5 retains the MHC class I in the Golgi apparatus (Marchetti et al., 2002). We wondered whether the reduction in surface N*01301-MHC class I was also due to the retention of residual complex in the Golgi. We performed two-colour confocal immunofluorescence in the various P815 cell lines where classical MHC class I were detected with mAb IL-A88 and a FITC-conjugated secondary antibody, and the Golgi apparatus was detected with Bodipy Ceramide-Texas Red. The cells were examined in a Leica TCS SP2 true confocal scanner microscope. The merge between FITC and Texas Red fluorescent signals was achieved using the Leica TCS SP2 accompanying software. The results showed that in all control cells, N*01301 was both associated with the Golgi membranes and clearly on the cell surface (figure 15a). In contrast, the cellular localisation of N*01301-MHC class I was markedly different in P815 cells expressing E5. Classical N*01301-MHC class I was totally associated with Golgi membranes and was not detectable on the cell surface by this technique (figure 15b). There was no difference between results obtained with P815-N*01301-pc4E5 cells and with P815-N*01301-pz4E5. These results confirm that BPV-4E5 cause a reduction of the surface expression of classical MHC I by retaining it in Golgi apparatus.

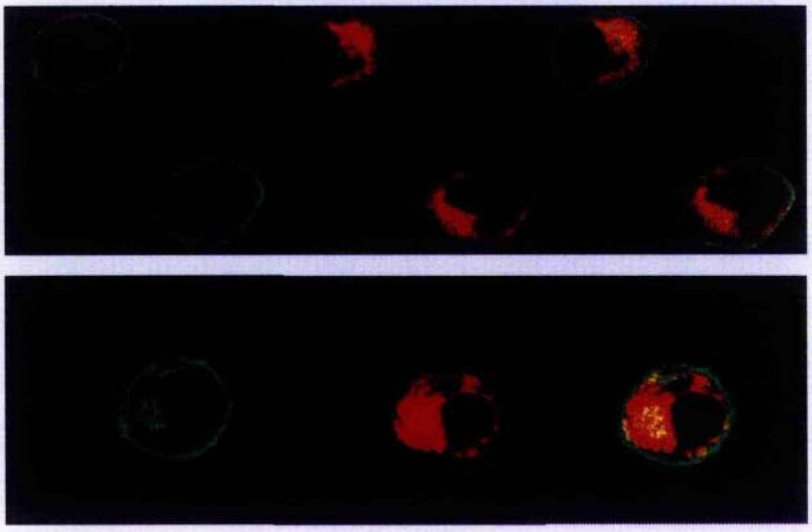
Figure 15. BPV-4E5 induces retention of N*01301 in Golgi apparatus of P815 cells. Two-colour confocal immunofluorescence was used to visualize Golgi apparatus and MHC class I. P815-N*01301 control cells (pcDNA and pZip) or cells expressing BPV-4 E5 (P815pc-4E5 and P815pZ-4E5) were stained with mAb IL-A88 and a FITC-conjugated secondary antibody, and the Golgi apparatus was detected with Bodipy Ceramide-Texas Red. The cells were examined in a Leica TCS SP2 true confocal scanner microscope. The merge between FITC and Texas Red fluorescent signals (orange) was achieved using the Leica TCS SP2 accompanying software. a) The cellular localization of N*01301 in control cells. b) The cellular localization of N*01301 in E5 cells. Single cells at the bottom of the panels are shown for greater detail. Cells in panel a-1, a-2 and cells in the lower panels of b-1 and b-2 were captured at magnification x400, cells in the upper panels of b-1 and b2 were captured at x200.

Figure 15 Localization of N*01301 in P815 cells expressing BPV-4E5

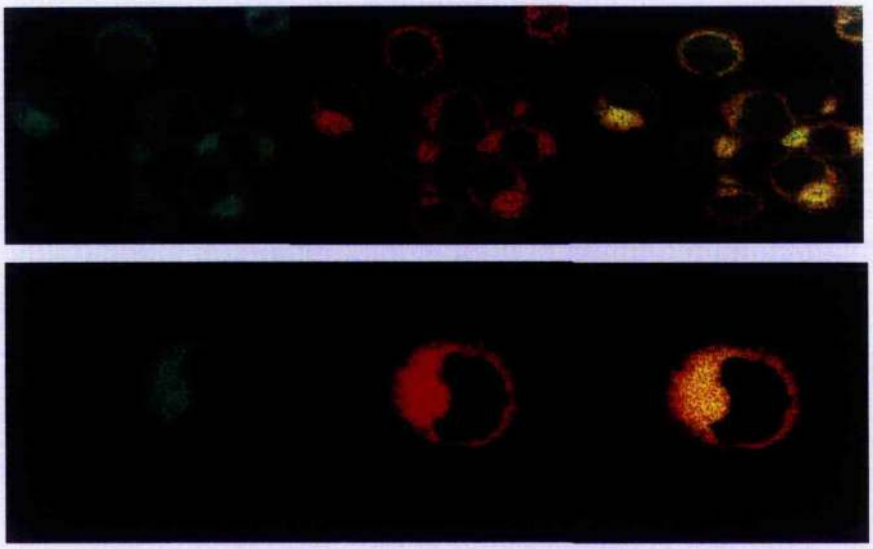
a-1 P815-N*01301pZip



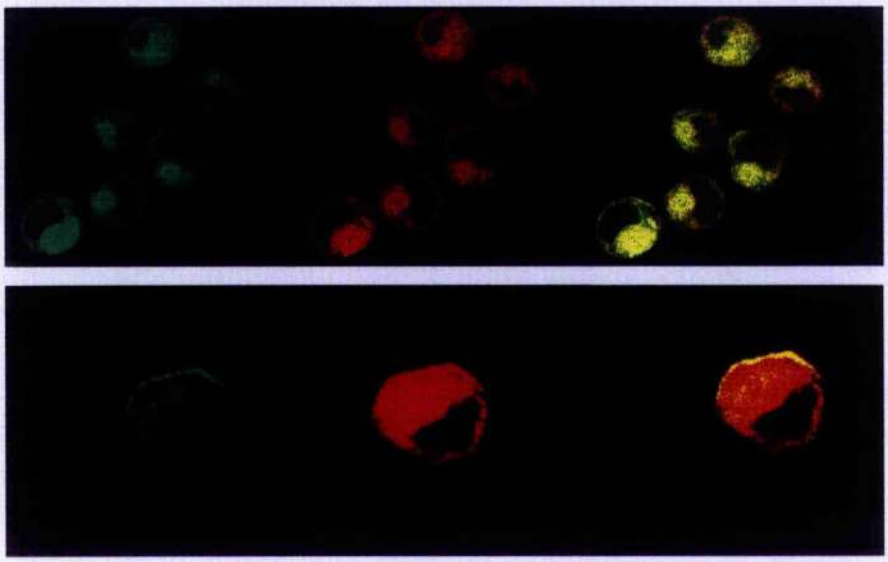
a-2 P815-N*01301pcDNA



b-1 P815-N*01301pc-4E5



b-2 P815-N*01301pZ-4E5

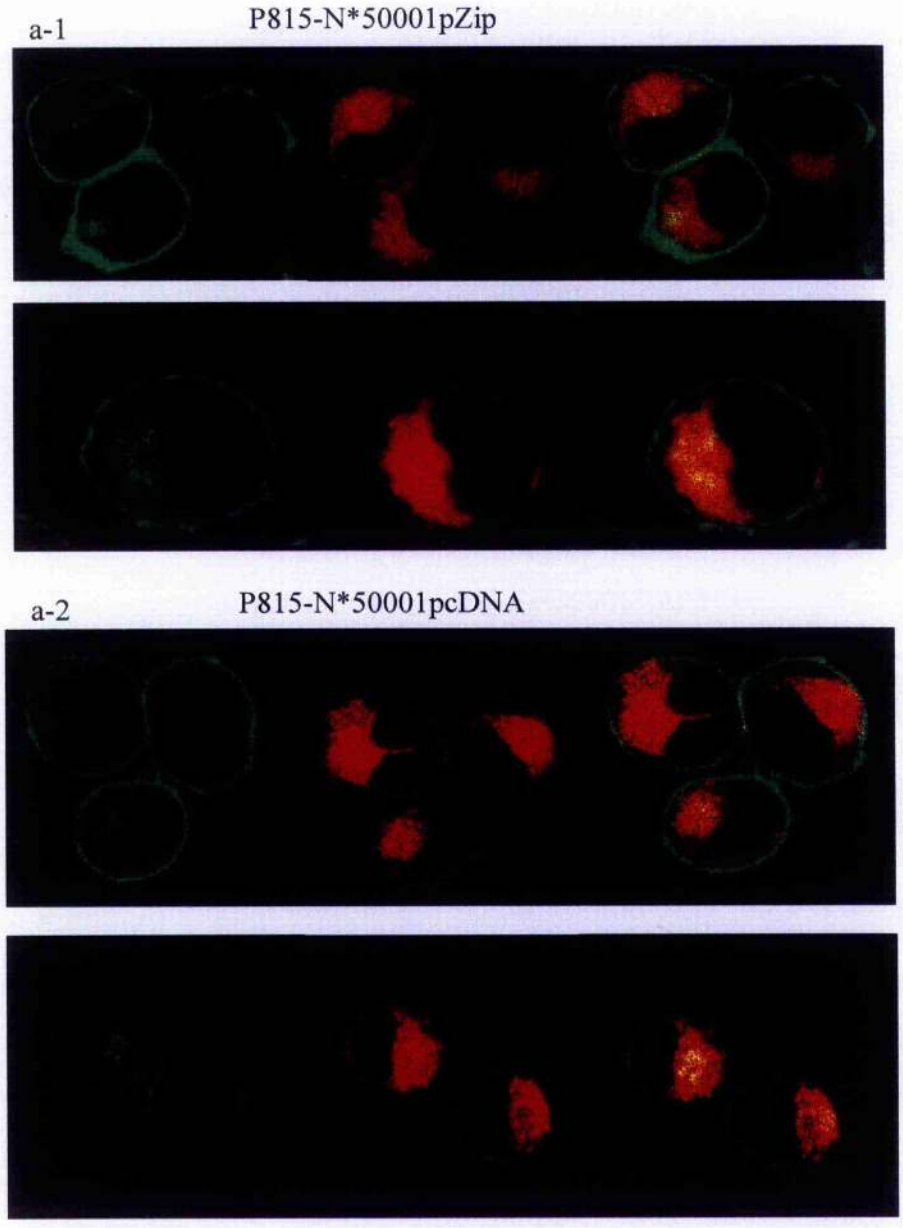


3.2.9 Localization of non classical MHC class I in P815 cells

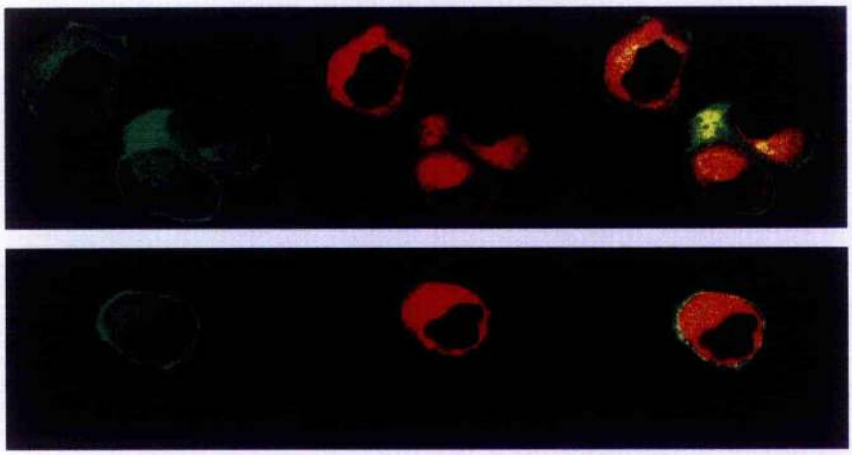
To determine the cellular localization of non-classical N*50001-MHC I, P815-N*50001 cells were stained with mAb IL-A88 and a FITC-conjugated secondary antibody and the Golgi apparatus was detected with Bodipy Ceramide-Texas Red. The cells were examined in a Leica TCS SP2 true confocal scanner microscope. The merge between FITC and Texas Red fluorescent signals was achieved using the Leica TCS SP2 accompanying software. The result showed that in E5 cells, N*50001-MHC I was clearly on the cell surface (figure 16b) and no different from control cells in its localization pattern both in Golgi apparatus and on the cell surface (figure 16a). There was no difference between results obtained with P815-N*50001-pc4E5 cells and with P815- N*50001-pZ4E5. These results show that BPV-4E5 does not interfere with transport of non classical MHC I to the cell surface.

Figure 16. BPV-4E5 does not retain N*50001 non classical MHC I in Golgi apparatus of P815 cells. Two-colour confocal immunofluorescenc was used to visualize Golgi apparatus and MHC class I. P815-N*50001 control cells (pcDNA and pZip) or cells expressing BPV-4 E5 (P815pc-4E5 and P815pZ-4E5) were stained with mAb H-A88 and a FITC-conjugated secondary antibody, and the Golgi apparatus was detected with Bodipy Ceramide-Texas Red. The cells were examined in a Leica TCS SP2 true confocal scanner microscope. The merge between FITC and Texas Red fluorescent signals (orange) was achieved using the Leica TCS SP2 accompanying software. a) The cellular localization of N*50001 in control cells. b) The cellular localization of N*50001 in E5 cells. Single cells at the bottom of the panels are shown for greater detail. Cells in the upper of a-1 and in the lower of a-2 and cells in b-1 and upper b-2 were captured at magnification x400, cells in the lower of a-1 were captured at x600, and cells in upper a-2 were captured x500 magnification and x300 magnification in lower b-2.

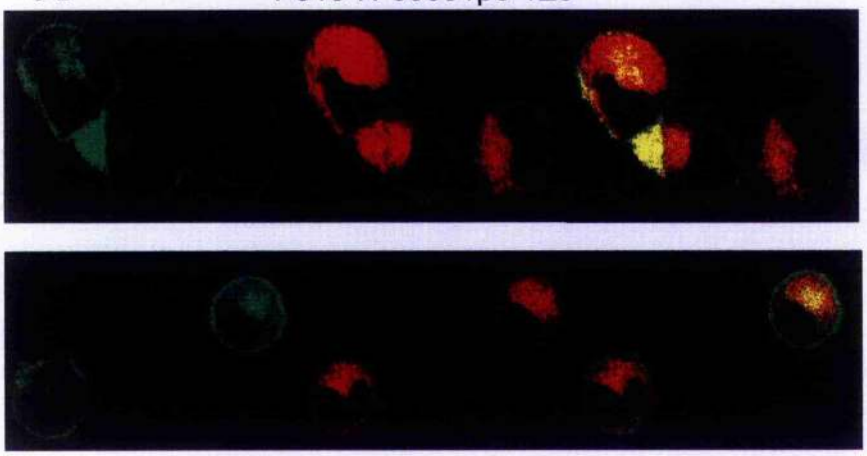
Figure 16 Localization of N*50001 in P815 cells expressing BPV-4E5



b-1 P815-N*50001pZ-4E5



b-2 P815-N*50001pc-4E5



3.2.10 BPV-4 E5 protein does not down-regulate bovine non-classical MHC class I in PalF cells.

To confirm the results obtained in P815 cells in which BPV-4 E5 does not down regulate bovine non classical (N*50001) MHC class I, primary embryonic bovine cells (PalF) transformed by papillomavirus oncogenes, with or without BPV-4 E5 as described by Jaggar et al (1990) were used. These cells express either BPV-4E5, BPV-4E7, HPV-16E6 and activated ras (PalF-4E5) or BPV-4E7, HPV-16E5 and activated ras (PalF-noE5). The *N*50001*cDNA was excised from pcDNA6-V5-His and sub-cloned in pFLAG expression vector. This plasmid was transiently transfected into PalF-4E5 and palF-noE5 cells and N*50001-MHC class I was detected with monoclonal anti-FLAG antibody and a FITC-conjugated secondary antibody, and the Golgi apparatus was detected with Bodipy Ceramide-Texas Red. The cells were examined in a Leica TCS SP2 true confocal scanner microscope. The merge between FITC and Texas Red fluorescent signals was achieved using the Leica TCS SP2 accompanying software. The result showed that in PalF-4E5 cells, N*50001 was clearly on the cell surface (figure 17b) and no different from the one in control (no E5) PalF cells in its localization pattern both in Golgi apparatus and on the cell surface (figure 17a). This result confirm that BPV-4E5 does not interfere with transport of N*50001-MHC class I to the cell surface. To confirm that BPV-4E5 does retain classical MHC I in the Golgi apparatus, an available GFP expression vector carrying human classical MHC I (B2705) (Marchetti et al 2002) was transiently transfected into PalF-4E5 or noE5 PalF cells. The result showed that in no E5 palF cells, in addition to its localization in the cytoplasm, B2705 was clearly on the cell surface (figure 17c-1). In contrast, in PalF E5 cells, the localization of B2705 was almost exclusively in the GA and no

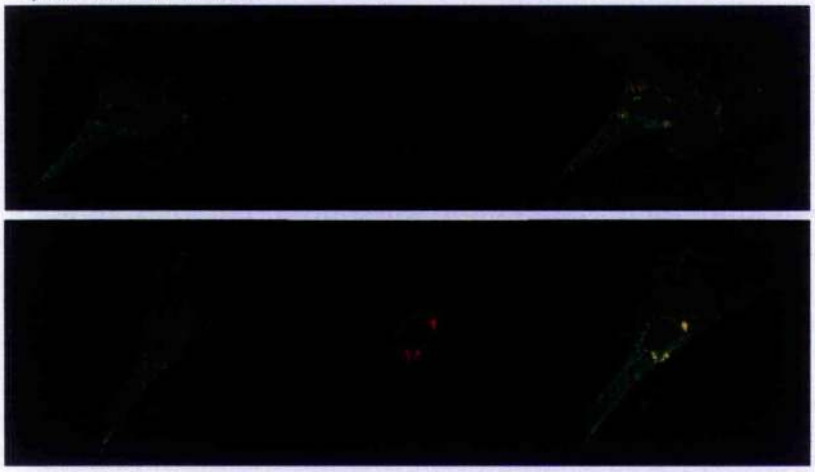
MHC could be detected on the cell membrane (figure 17c-2). It is to be noted that the Golgi apparatus is deformed in PalF-4E5 cells as described by (Ashrafi et al 2002; Marchetti et al 2002). Furthermore, the morphology of Golgi apparatus was abnormal in E5 PalF cells. This result confirms in agreement with (Ashrafi et al 2002) that down-regulation of MHC I by BPV-4E5 is species independent.

Figure 17. BPV-4 E5 does not retain N*50001 MHC class I in Golgi apparatus of PalF cells. *N*50001 cDNA* was cloned into pFLAG expression vector and transfected into PalF-4E5 and PalF-noE5 cells. N*50001-MHC class I was detected with monoclonal anti-FLAG antibody and a FITC-conjugated secondary antibody, and the Golgi apparatus was detected with Bodipy Ceramide-Texas Red. The cells were examined in a Leica TCS SP2 true confocal scanner microscope. The merge between FITC and Texas Red fluorescent signals was achieved using the Leica TCS SP2 accompanying software. a) Shows the localization of N*50001 in PalF-no E5 cells; b) Shows the localization of N*50001 in PalF-4E5 cells; c) shows the localization of B2705 human classical MHC I in PalF-4E5 and PalF-noE cells. Cells in panels (a, b and c) were captured at x800 magnification.

Figure 17 BPV-4E5 does not downregulate surface expression of N*50001 in PalF cells

a) PalF no E5-N*50001

b) PalFE5-N*50001



C-1 PalF-no E5

C-2 PalF-4E5

3.3.1 Detection of classical and non-classical MHC I heavy chains in P815 cells

The results above show that BPV-4 E5 causes a reduction in the level of total N*01301-MHC class I, but not of N*50001-MHC I. To confirm this observation, western blot experiments were performed on protein extracts from N*01301 and N*50001-MHC I in P815 cells either expressing E5 or carrying empty vector. The membranes were incubated with mAb IL-A88, which detects all bovine MHC I heavy chains. The result showed that there was less N*01301 expressed in P815-N*01301-pc4E5 or P815-N*01301-pZE5 cells than in the control cells (Figure 18a); on the contrary, the expression of N*50001 was unchanged in P815-N*50001-pc4E5 or P815-N*50001-pZ4E5 cells, although in control cells it was lower than that of N*01301. In agreement with (Ashrafi et al 2002; Marchetti et al 2002), these results confirm that BPV-4E5 in contrast to HPV-16E5 causes a remarkable reduction both surface and total MHC I.

3.3.2 BPV-4 E5 protein causes degradation of bovine classical MHC I heavy chain but not non-classical heavy chain in P815 cells.

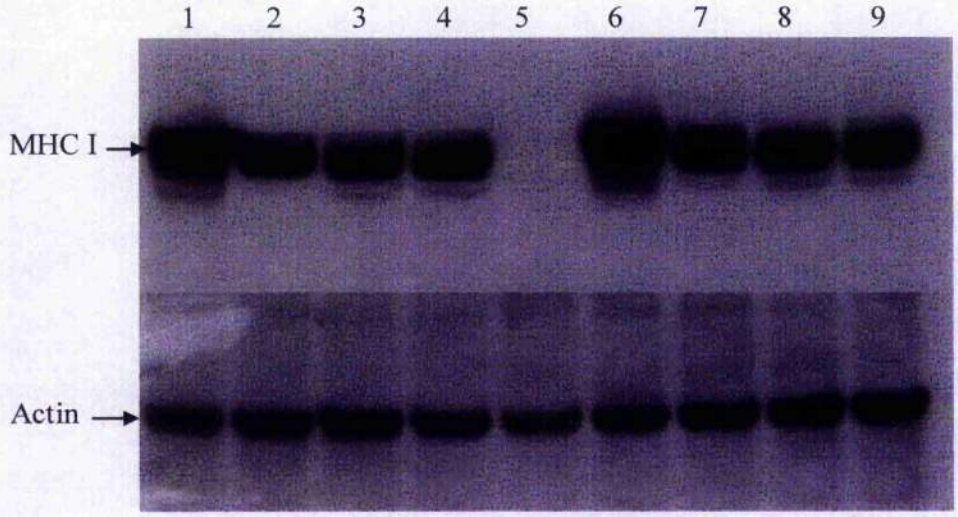
To investigate if the reduction of the total amount of MHC classes I in E5 cells is due to protein degradation and also to investigate whether E5 affects non-classical MHC class I protein stability. Control cells and E5 expressing P815 cells were treated with cyclohexamide to allow quantification of protein stability in E5 cells. Cyclohexamide is an inhibitor of protein biosynthesis as it blocks translational elongation, therefore allowing analysis of half-life of pre-existing proteins. Treated cells were harvested after 1, 2, 5, 10, and 24 hours of cyclohexamide treatment and protein samples were extracted, elctrophoresed and transferred on nitrocellulose membranes as described in section 2.2.45. For detection of MHC I, membranes

were incubated with mouse mAb IL-A88, for detection of actin as control with mAb AB-1 as described in section 2.2.47. Similar experiments were performed on protein extracts from cells treated with DMSO, the solvent for cyclohexamide. In control cells, N*01301 was stable and could still be detected after 24 hours of treatment (figure 18b-1); in remarkable contrast, in the 4E5-expressing cells N*01301 was unstable and was practically undetectable after 10 hours of treatment (figure 18b-2). There was no difference between the stability of N*50001 in control or 4E5-expressing cells; in both cases N*50001 HC was still detectable after 24 hours of treatment (figure 18b-3,4), comparable to that of N*01301 in control cells. There was no effect of DMSO on N*50001 or N*01301 heavy chain stability either in control or 4E5-expressing cells (figure 18c). These results show that BPV-4 E5 causes degradation of classical MHC I heavy chain. The results also show that E5 does not disrupt expression, half-life and transport of non-classical N*50001-MHC class I. It has been shown in PalF cells that BPV-4E5 inhibits MHC 1 heavy chain gene transcription (Marchetti et al 2002). The effect of E5 on transcription of MHC I HC could not be investigated in this system because MHC I is transcribed under control of immediate early (IE) promoter of cytomegalovirus.

Figure 18. BPV-4 E5 induces degradation of classical N*01301 MHC class I heavy chain but not non-classical N*50001 heavy chain. For detection of N*01301 and N*50001 MHC class I, the membranes were incubated with the mAb IL-A88 specific for MHC class I heavy chain and mAb AB-1, specific for β-actin. a) Steady state levels of classical N*01301 heavy chain in P815 control cells (pcDNA and pZip) lane 1 and lane 6 respectively. There is a reduction in N*01301 heavy chain in E5 cells (pc-4E5 lane 2 and pZ-4E5 lane 7). There is no difference between levels of non-classical N*50001 heavy chain in P815 control cells (pcDNA lane 3 and pZip lane 8) and cells expressing E5 (pc-4E5 lane 4 and pZ-4E5 lane 9), but there are comparable amounts of β-actin in all cell lines. b) Half-life measurement of classical N*01301 and non-classical N*50001 heavy chain in cyclohexamide-treated P815 control cells and cells expressing E5 cells, panel (1) represents N*01301 HC in control cells; panel (2) represents N*01301 HC in E5 cells; panel (3) represents N*50001 HC in control cells and panel (4) represents N*50001 HC in E5 cells. N*01301 HC in E5 cells was detected up to approximately 5 hours and disappeared after 10 hours of treatment whereas N*50001 HC can still be detected after 24 hours of treatment as indicated by red circles. c) P815 control cells and cells expressing E5 were treated with DMSO for different periods of time (1-24 hours), panel (1) represents N*01301 HC in control cells; panel (2) represents N*01301 HC in E5 cells.

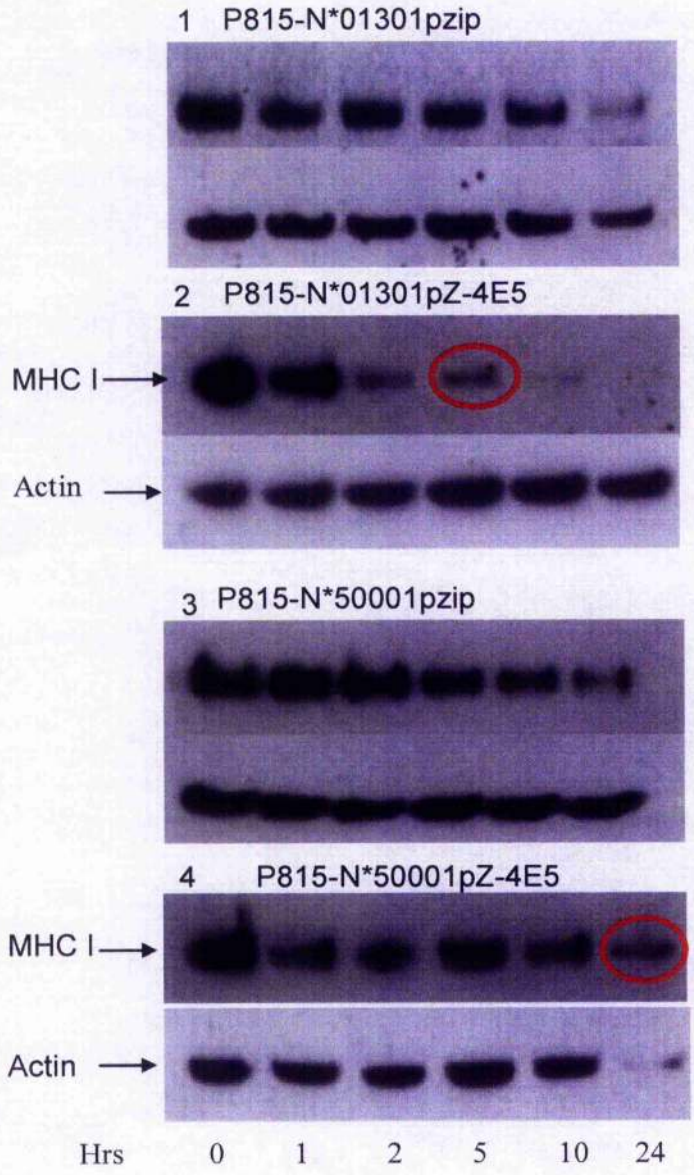
Figure 18

a- BPV-4E5 reduces expression of N*01301 heavy chain

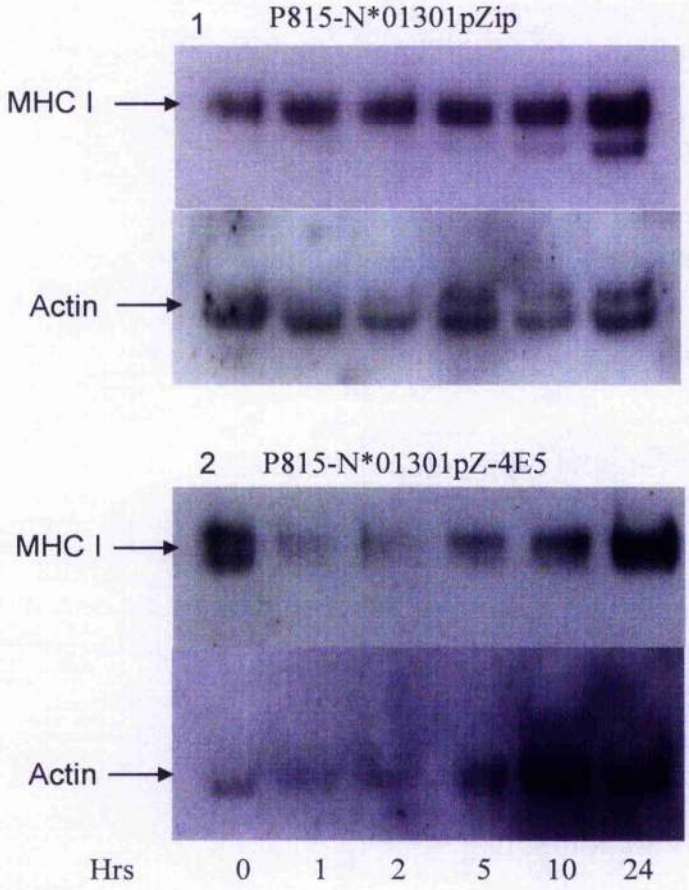


1= P815-N*01301pcDNA
2= P815-N*01301pc-4E5
3= P815-N*50001pcDNA
4= P815-N*50001pc-4E5
5= P815
6= P815-N*01301 pZip
7= P815-N*01301Z-4E5
8= P815-N*50001 pZip
9= P815-N*50001 pZ-4E5

b) BPV-4E5 causes degradation of N*01301 heavy chain



c) Protein blots from treated cells with DMSO as control

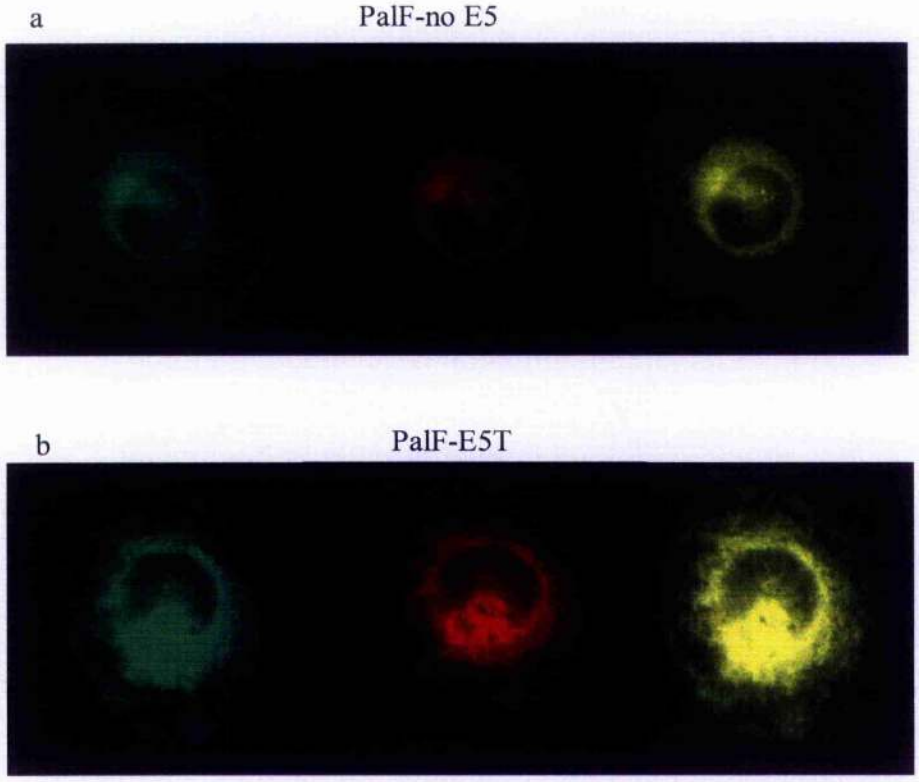


3.4 The C-terminal domain of BPV-4 E5 is responsible for down-regulation of MHC class I

The C-terminal tail of E5 has an integral role in the function of the protein. The C terminus truncated form of E5 (E5T), comprises only the first 32 amino acid residues (O'Brien et al 1999). E5T is not transforming, does not induce the typical morphological changes brought about by E5, does not distort the GA and does not down-regulate MHC I (Ashrafi et al 2000; Ashrafi et al 2002). E5 physically interacts with MHC class I heavy chain and this interaction is mediated by the C terminal tail of E5 (Marchetii *et al.*, 2006) indicating that interaction between E5 and HC is partially responsible for down-regulation of MHC I. To determine whether the C-terminus tail of E5 is responsible for retention of MHC I in the Golgi apparatus, PalF cells expressing 4E5T or not were incubated with BODIPY ceramide TR for Golgi visualization. For detection of MHC I, cells were incubated with mAb IL-A88 and antimouse IgG FITC-conjugated secondary antibody. The cells were examined in a Leica TCS SP2 true confocal scanner microscope. The merge between FITC and Texas Red fluorescent signals was achieved using the Leica TCS SP2 accompanying software. The result showed that the localization of MHC I on the cell surface was not disrupted in cells expressing E5T (figure19b), and was similar to control cells, being found both in the Golgi apparatus and on the cell surface (figure 19a). In agreement with (Ashrafi et al 2002; Marchetti et al 2002), these results confirm that the C-terminal tail of BPV-4E5 is responsible for down-regulation of MHC I.

Figure 19. Surface localization of MHC class I in control PalF cells (a), and in cells expressing BPV-4 E5T (b). MHC class I was detected with mAb IL-A88 and FITC-conjugated secondary antibody (green) and the Golgi apparatus with BODIPY-TR-ceramide (red) in a Leica TCS SP2 confocal scanner microscope. The merge between the FITC and Texas Red fluorescent signals was achieved using the Leica TCS SP2 accompanying software. Cells in panels (a and b) were captured at x1,200 magnification.

Figure 19 The C-terminal tail of BPV-4E5 is essential for down-regulation of MHC I



3.5 HPV-16 E5 protein down-regulates human MHC class I (HLA) molecules

Mouse mastocytoma P815 cell lines were extremely useful in the investigation of the effects of BPV-4 E5 on bovine classical and non classical MHC class I, since classical and non classical MHC I can not be distinguished in bovine cells. Furthermore, P815 cells provided definite proof that E5 can interfere with classical MHC class I pathways by itself. Down-regulation of classical HLA-A and B by HPV-16 E5 protein, but not of HLA-E and HLA-C, has been reported in immortalized human keratinocytes (Ashrafi et al 2005). To investigate the effect of HPV-16E5 on the expression of HLA-A2 and HLA-E separately from each other and from other HLA molecules, as mAb DT9 does not distinguish between HLA-C and HLA-E, P815 cells were transfected with pc16E5 prior to introducing HLA-E or HLA-A2.

3.5.1.1 Introduction of HPV-16E5 into P815 cells

Parental P815 cells were stably transfected with 4μg of either pcDNA or pc16E5 by using Nucleofector (Amaxa- Germany) according to manufacturer instructions. Positive selection of transfectants was achieved by growing cells in medium containing G418 as described in section 2.2.19.

3.5.1.2 HPV-16 E5 expression in P815 cells

Like BPV-4 E5, HPV-16 E5 is expressed at low levels in cells and there is no dependable antibody for detection of E5 protein. Therefore, we investigated the transcription of the E5 ORF in P815 cells using quantitative RT-PCR. RNA was isolated, purified from any residual DNA and typically 1μg of purified RNA was used for each sample. We analysed two pc-16E5 representing two P815 pools for

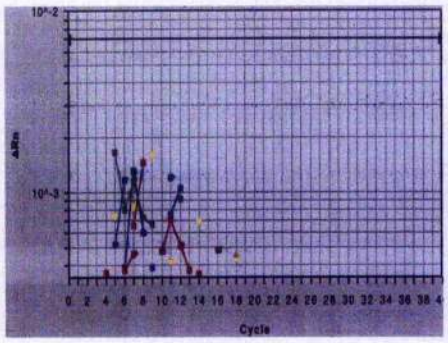
the presence of E5 transcripts. Probes and primers for HPV16 E5 and mouse β-actin were designed using the primer Express Software programme (refer to page 103). In each experiment, additional reactions, with 10 fold serial dilutions of template DNA, were performed with each set of primers and probes on the same 96-well plates to generate standard curves. Quantitive RT-PCR was used to determine the relative amount of HPV-16 E5 and β-actin mRNA in triplicate experiments of each E5 transfected cell line and from cells carrying empty vectors by using a standard curve (figure 20a). The results show that all of the transfected cells (pc16E5) express the E5 gene but at 10,000 fold less than β-actin (figure 20b). As expected, no E5 RNA was detected in the control cells. This low level of E5 expression is in agreement with, Ashrafi et al, 2005, in all HPV-16 E5-transfected HaCaT, NIH 3T3 and bovine PalF cell lines.

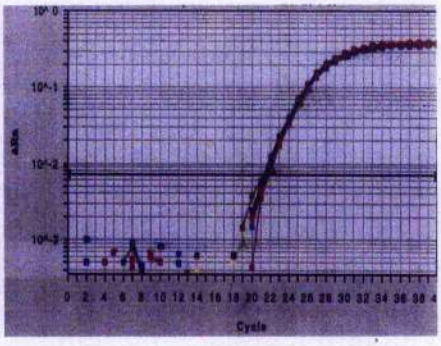
Figure 20. Taq-Man RT-PCR was used to determine the expression of E5 mRNA in the transfected P815 cells. a) The expression of HPV-16 E5 RNA and actin mRNA was determined in P815-pcDNA cells or cells expressing HPV-16 E5 (pc16E5), (two pools of pc16E5). b) The histograms represent the average with standard deviation of four independent measurements. Note that the levels of E5 RNA are four orders of magnitude lower than those of β-actin RNA.

Figure 20

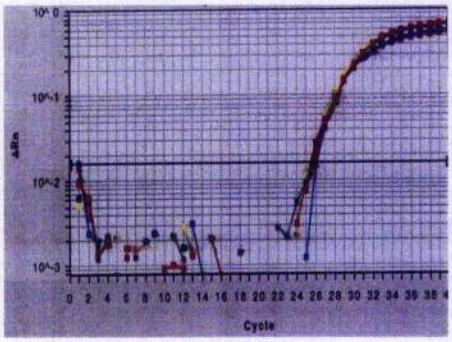
a) Expression of HPV-16 E5 RNA in transfected P815 cells

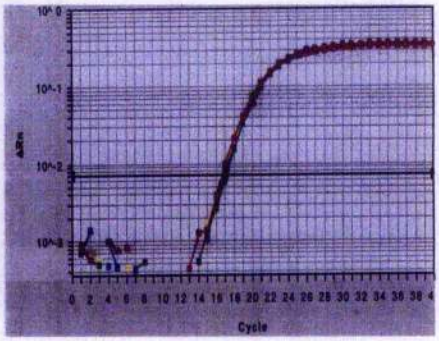
P815 pcDNA





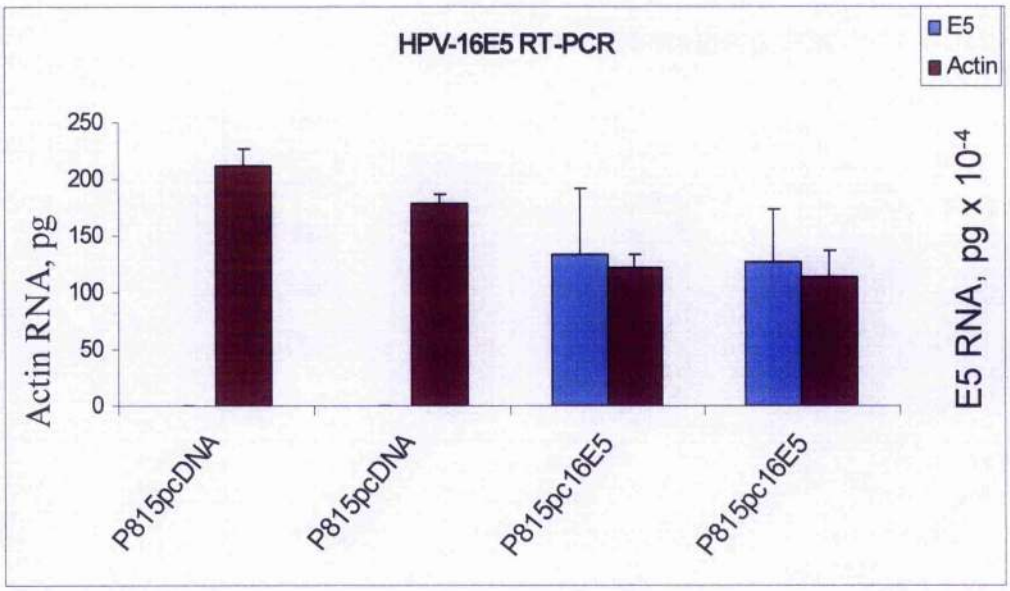
P815pc16E5





E5 Actin

b) Quantitative HPV-16E5 and actin RNA in transfected P815 cells



3.5.1.3 Introduction of HLA-A2 cDNA into P815-16E5 cells

To determine the effect of HPV E5 on expression of HLA-A2, HLA-A2 cDNA was firstly excised from the pBLuescript II SK (+) plasmid using digestion enzymes and recloned in pcDNA6 plasmid as described in section 2.2.21. Secondly, P815 cells carrying empty vector or cells expressing 16E5 were transfected with HLA-A2 cDNA using Nucleofector (Amaxa- Germany) according to manufacturer instructions. Transfectants were selected in medium containing blasticidin as described in section 2.2.21.

3.5.1.4 Introduction of HLA-E cDNA into P815-16E5 cells

To determine the effect of HPV E5 on expression of HLA-E, HLA-E cDNA was first amplified by PCR from the pBLuescript II SK (+) plasmid utilizing the primers described on page 89 of this thesis, (section 2.2.20). The PCR products were sub-cloned into pcDNA6 plasmid as described on page 90 (section 2.2.20). Secondly, P815 cells carrying empty vector or cells expressing 16E5 were transfected with HLA-E cDNA using Nucleofector (Amaxa- Germany) according to manufacturer instructions. Transfectants were selected in medium containing blasticidin as described in section 2.2.20. There was no need to co-transfect the human β_2 microglobulin (β_2m) gene with the HLA-A2 and HLA-E HC genes into P815 cells because the human class I HC associates with mouse β_2m. However, it is not known whether human HLA-A2/E HCs exchange mouse β_2m with bovine β_2m provided by the foetal calf serum in the culture medium.

3.5.2 Expression of HLA in cells expressing HPV-16E5

The effect of E5 on MHC class I was investigated by FACS, immunofluorescence and western blots. FACS was used to determine the surface and total expression of HLA class I, immunofluorescence to show the cellular localization of HLA class I, and western blots to show the expression of HLA class I heavy chains in P815 cells carrying empty vectors or cells expression HPV-16 E5.

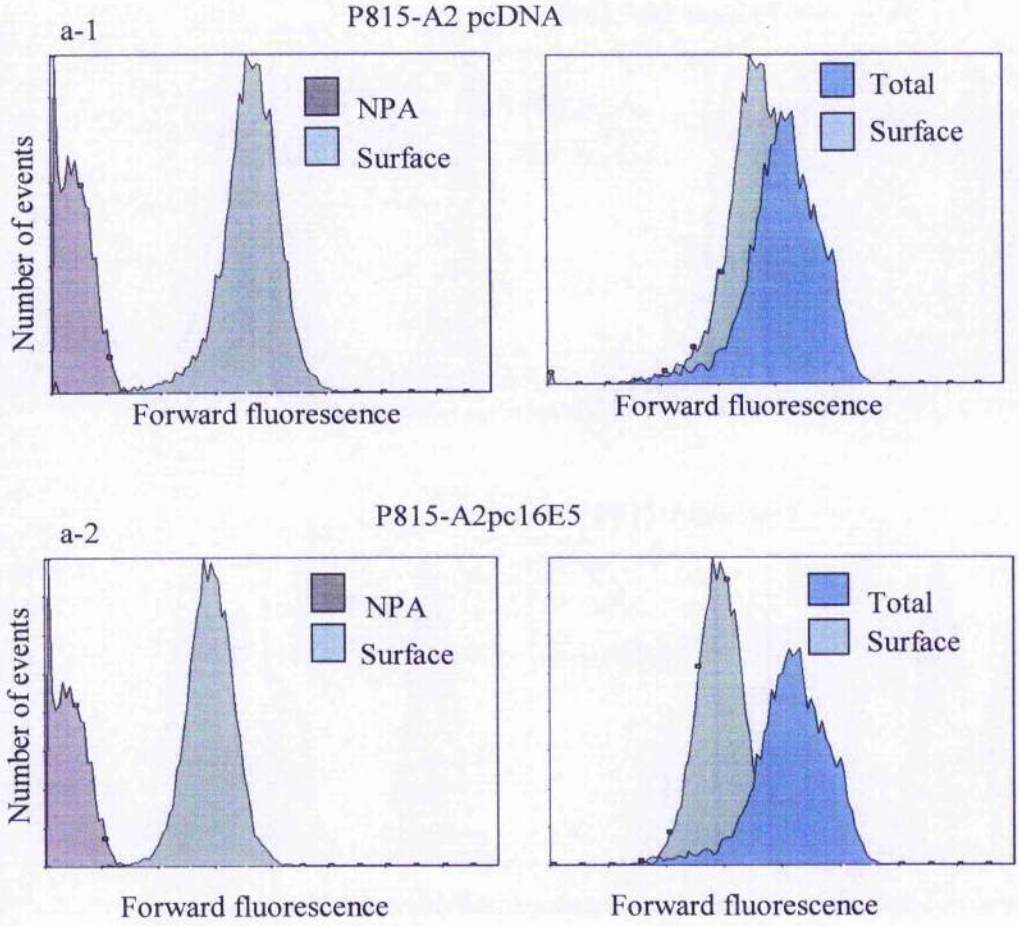
3.5.2.1 Detection of HLA-A2 by FACS analysis

It has been shown that HPV-16 E5 causes the retention of HLA-A/B in the Golgi apparatus and prevents its export to the plasma membrane of immortalized HaCaT cells (Ashrafi et al 2005). We extended these observations to investigate the effect of HPV-16E5 on HLA-A2 separately from HLA-B molecules. P815-HLA-A2 cells harbouring the empty vector pcDNA or expressing HPV-16E5 were incubated with anti-HLA class I mAb W6/32, analysed for levels of both surface and total HLA class I. The results showed that there was no fluorescence shift from parental P815 cells stained with mAb W6/32 or from P815-HLA-A2 stained with secondary antibody only. In control cells, P815-HLA-A2-pcDNA, there was a considerable fluorescence shift representing the surface expression of HLA-A2 (figure 21a-1). In contrast, in P815-HLA-A2 expressing 16E5, there was a remarkable reduction in fluorescence shift for surface HLA (figure 21a-2) compared with control cells. There was no difference in fluorescence shift for total HLA-A2 between control and cells expressing 16E5. To quantify the level of surface and total HLA-A2 in E5 cells, at least four experiments were performed and the mean of forward fluorescence was calculated. The results showed that P815-HLA-A2 expressing HPV-16E5 had approximately 50 % lower surface HLA-A2 than control cells. In

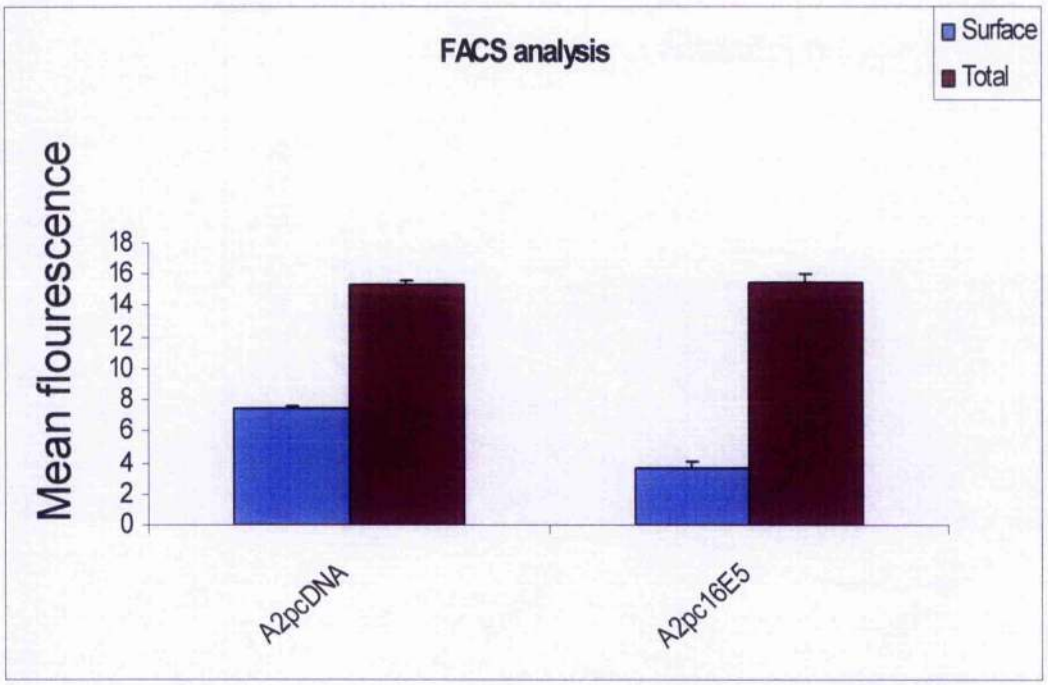
contrast, there was no significant difference in total HLA-A2 level between control and cells expressing HPV-16E5 (figure 21b). These results confirm that HPV-16E5 reduces the level of surface HLA-A2 but does not affect its total amount.

Figure 21. Down-regulation of HLA-A2 on the cell surface was investigated in P815 cells expressing HPV-16 E5. P815-HLA-A2-pcDNA cells or P815-HLA-A2 cells expressing HPV-16 E5 were stained with mAb W6/32 and analysed for expression of surface and total HLA-A2 by FACS. a) Example of flow cytometry profiles of surface and total HLA-A2 in P815 control cells or cells expressing E5. b) The mean forward fluorescence with standard deviation calculated from the flow cytometry profiles of at least four independent experiments performed on two cell lines.

Figure 21 HPV-16E5 down-regulates surface expression of HLA-A2



b

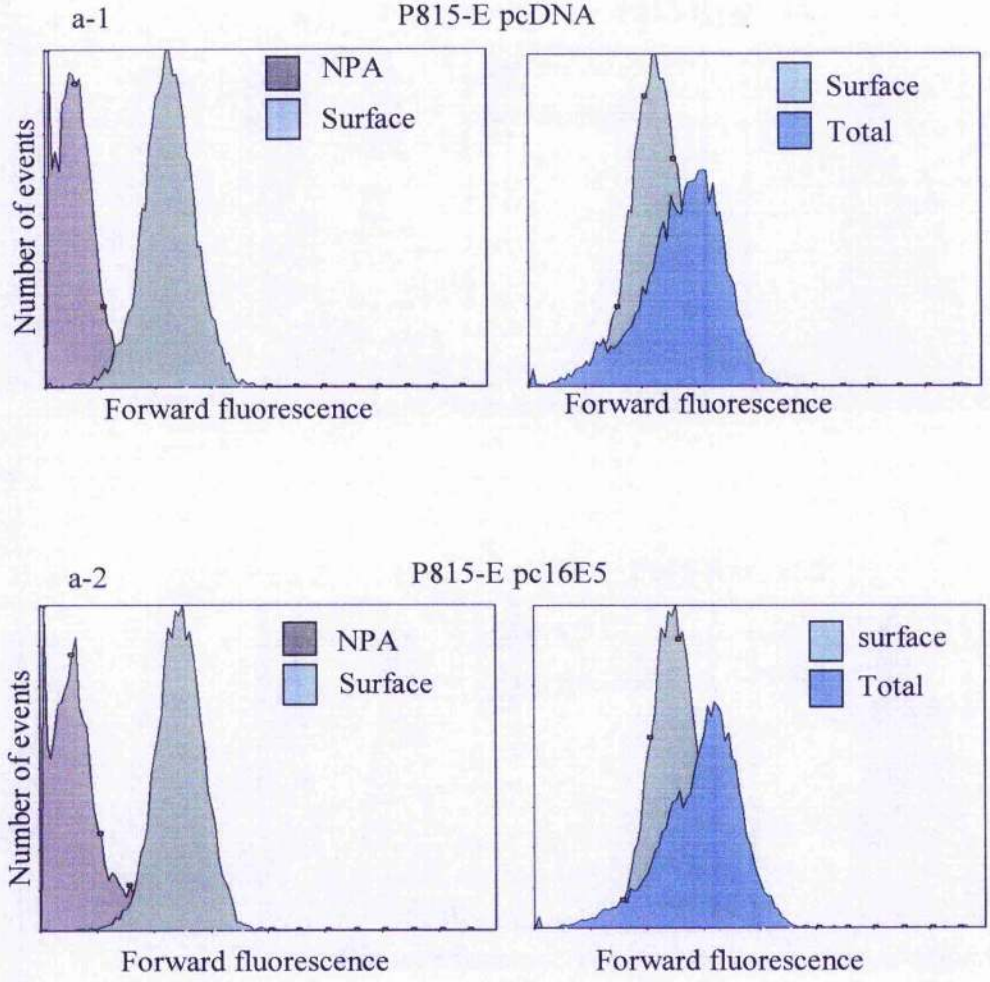

FACS analysis
Surface
Total
Mean flourescence
18
16
14
12
10
8
6
4
2
0
A2pcDNA
A2pc16E5

3.5.2.2 Detection of HLA-E by FACS analysis

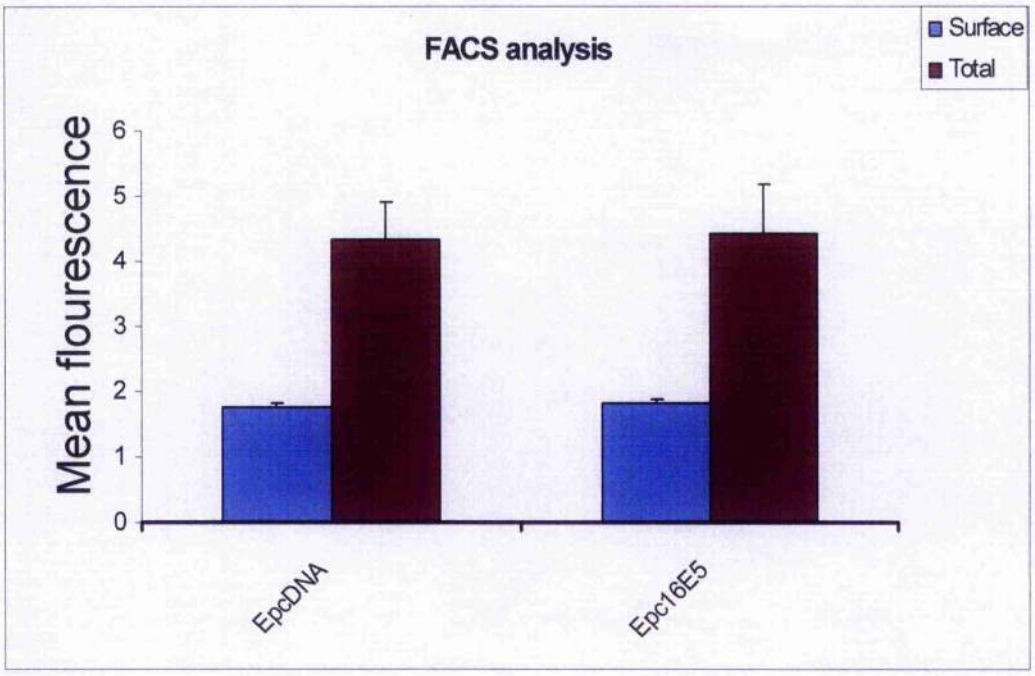
It has been reported that HPV-16E5 does not interfere with surface expression of HLA C/E in E5-transformed HaCaT cells by using mAb DT9. This antibody does not distinguish between classical HLA-C and non classical HLA-E, hence we investigated the specific effect of HPV-16E5 on HLA-E in P815 cells expressing only HLA-E. P815-HLA-E-pcDNA or P815-HLA-E-16E5 cells were incubated with mAb DT9 and analysed for levels of both surface and total HLA-E. The results showed that there was no significant difference in fluorescence shift for surface HLA-E in the two cell lines (figure 22a-1, 22a-2). Likewise, there was no difference between two cell lines in the total HLA-E. To quantify the level of surface and total HLA-E in E5 cells, at least four experiments were performed and the mean of forward fluorescence shift was calculated. The levels of surface and total of HLA-E were unaffected in cells expressing HPV-16E5 and were similar to the levels of surface and total HLA-E in control cells (figure 22b). These results confirm that HPV-16E5 does not down-regulate non-classical HLA-E.

Figure 22. HPV-16E5 does not down-regulate non-classical MHC I on the cell surface in the transfected P815 cells. P815-HLA-E-pcDNA or P815-HLA-E-pc16E5 were stained with mAb DT9 and analysed for expression of surface and total HLA-E by flow cytometry. a) Example of flow cytometry profiles of surface and total HLA-E in P815 control cells (a-1) or cells expressing E5 (a-2). b) The mean forward fluorescence with standard deviation calculated from the flow cytometry profiles of at least four independent experiments performed on two cell lines.

Figure 22 HPV-16E5 does not down-regulate surface expression of HLA-E



b

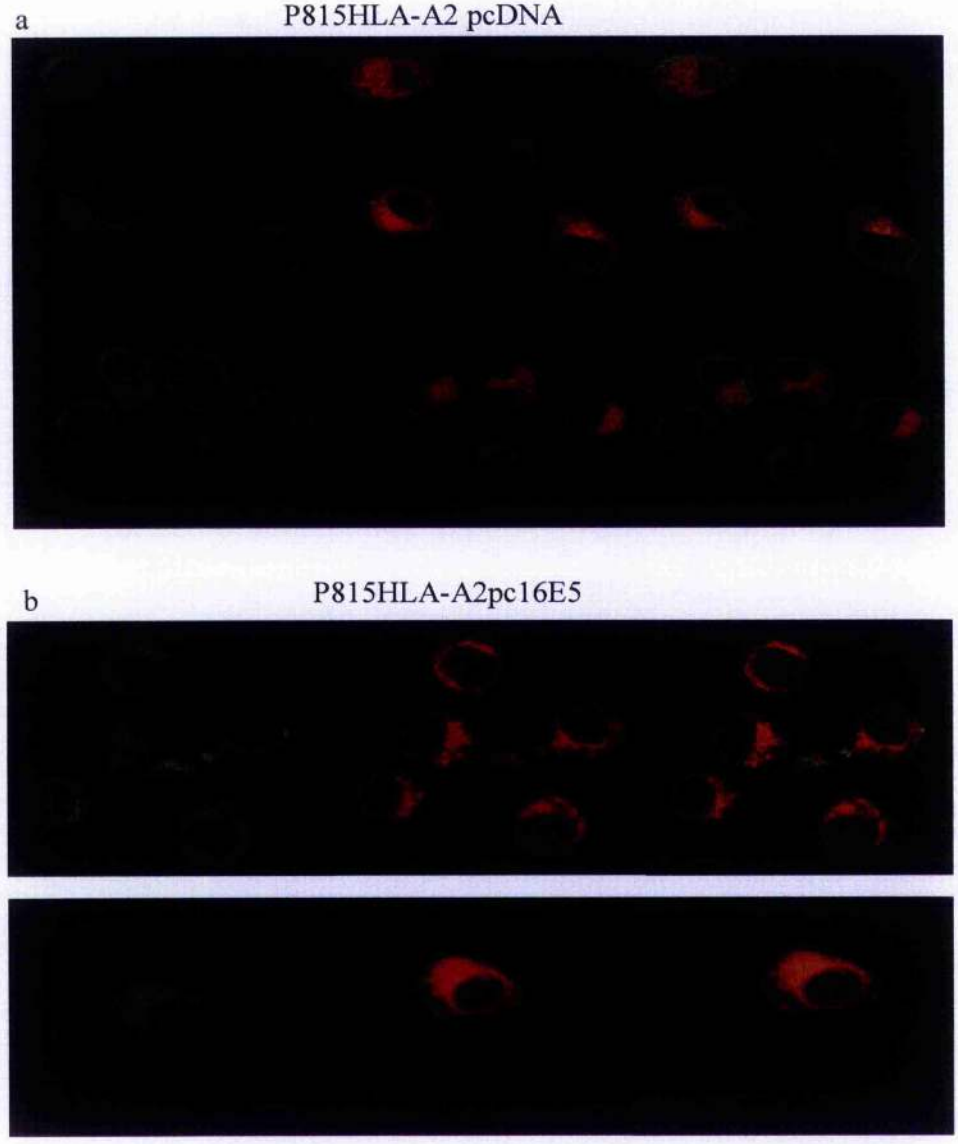

FACS analysis
Surface
Total
Mean flourescence
6
5
4
3
2
1
0
EpcDNA
Epc16E5

3.5.2.3 Detection of HLA-A2 by immunofluorescence

In HaCaT-16E5 cells, E5 retains the HLA-A and B in the Golgi apparatus (Ashrafi et al 2005). We wondered whether the reduction in surface HLA-A2 in P815 cells was also due to the retention of the complex in the Golgi. To investigate the cellular localization of HLA-A2, we performed two-colour confocal immunofluorescence in two P815 cell lines. The cell pellet was resuspended in fixing solution for 10 min, washed twice in PBS and seeded in poly-L-lysine-pre-treated eight-well slide at 2x 10^4 cells/well following the immunofluorescence staining protocol. P815-HLA-A2-pcDNA or P815-HLA-A2-pc16E5 cells were stained with mAb W6/32 and a FITC-conjugated secondary antibody, and the Golgi apparatus was detected with Bodipy Ceramide-TR. In pcDNA cells, HLA-A2 was detected both on the cell surface and in the Golgi apparatus (figure 23a) whereas, in cells expressing HPV-16E5, HLA-A2 was only detected in the Golgi apparatus (figure 23b). The result confirms that HPV-16E5 retains HLA-A2 in the Golgi apparatus and prevents its transport to the cell surface.

Figure 23. HPV-16E5 induces retention of HLA-A2 in Golgi apparatus in P815 cells. Two-colour confocal immunofluorescence. P815-HLA-A2-pcDNA cells or cells expressing HPV-16 E5 were stained with mAb W6/32 and a FITC-conjugated secondary antibody, and the Golgi apparatus was detected with Bodipy Ceramide-Texas Red. The merge between FITC and Texas Red fluorescent signals (orange) was achieved using the Leica TCS SP2 accompanying software. a) The cellular localization of HLA-A2 in control cells. b) The cellular localization of HLA-A2 in cells expressing E5. Single cells at the bottom of panel (b) are shown for greater detail. Cells in panel (a) were captured at x200, and x300 magnification for cells in panel (b).

Figure 23 Cellular localization of HLA-A2 in P815 cells

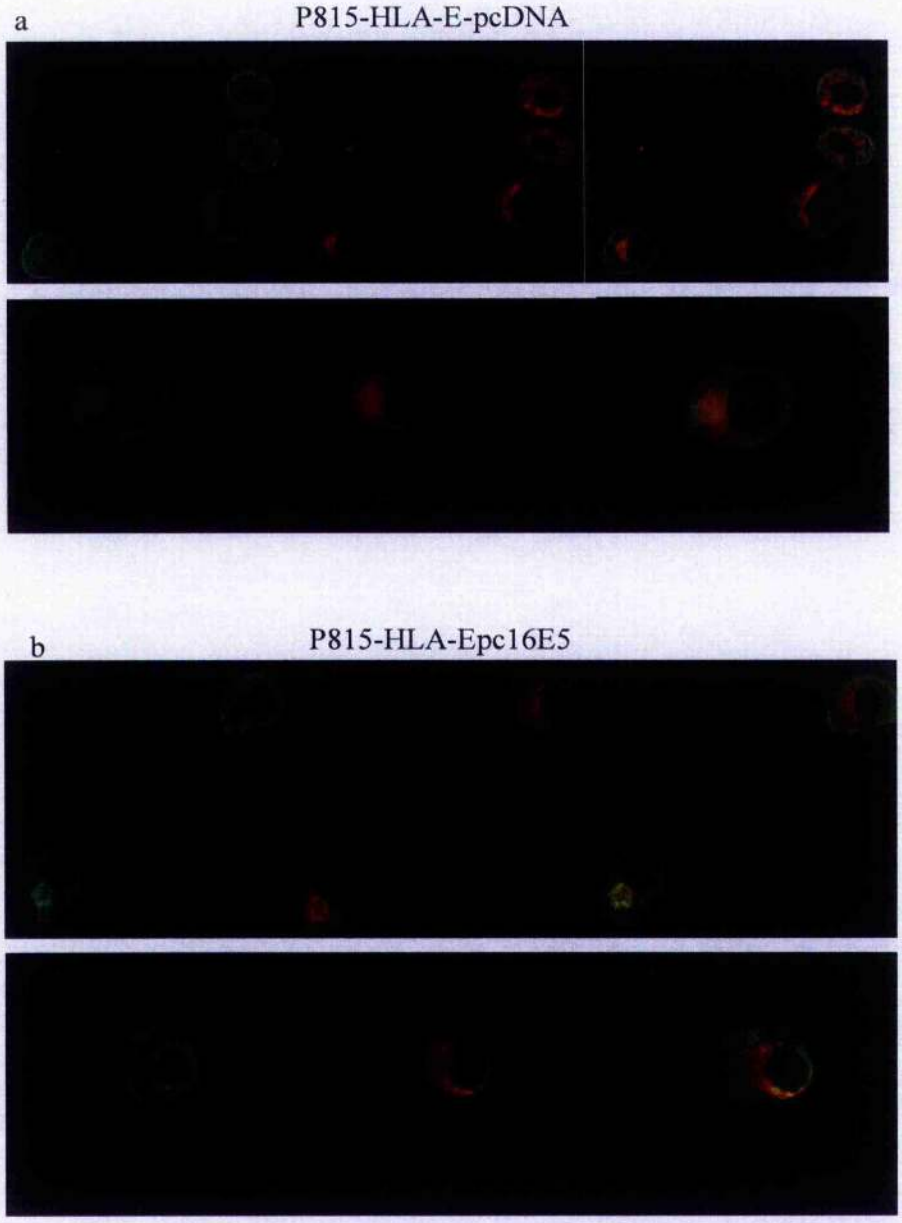


3.5.2.4 Detection of HLA-E by immunofluorescence

To investigate the cellular localization of HLA-E, we performed two-colour confocal immunofluorescence in two P815 cell lines. P815/HLA-E pcDNA or P815/HLA-E pc16E5 cells were incubated with anti-HLA-C/E mAb DT9 and a FITC-conjugated secondary antibody, and the Golgi apparatus was detected with Bodipy Ceramide-T R. The result showed that there was no significant difference between empty vector and 16E5 P815 cells in their expression pattern of HLA-E both on the cell surface and in the Golgi apparatus (figure 24a,b). These results confirm that HPV-16E5 does not interfere with the transport of HLA-E to the cell surface.

Figure 24. HPV-16 E5 does not retain HLA-E in Golgi apparatus in P815 cells. Two-colour confocal immunofluorescence. P815-HLA-E-pcDNA cells or cells expressing HPV-16 E5 were stained with mAb DT9 and a FITC-conjugated secondary antibody, and the Golgi apparatus was detected with Bodipy Ceramide-Texas Red. The merge between FITC and Texas Red fluorescent signals (orange) was achieved using the Leica TCS SP2 accompanying software. a) The cellular localization of HLA-E in control cells. b) The cellular localization of HLA-E in cells expressing E5. Single cells at the bottom of the panels are shown for greater detail. The top of panels (a) and (b) are at x200 magnification, the bottom of the panels (a) and (b) are at x400 magnification.

Figure 24 Cellular localization of HLA-E in P815 cells

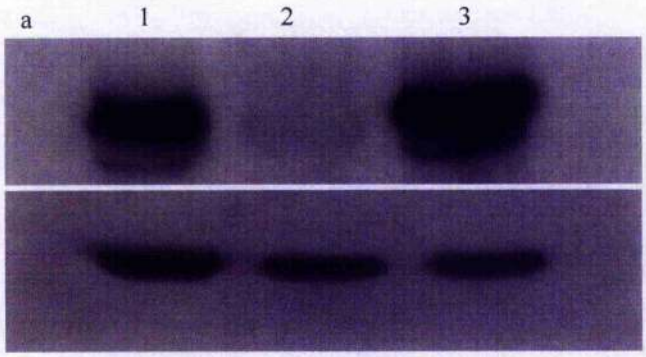


3.5.2.5 Expression of HLA-A2 or HLA-E heavy chains is not inhibited by HPV-16E5

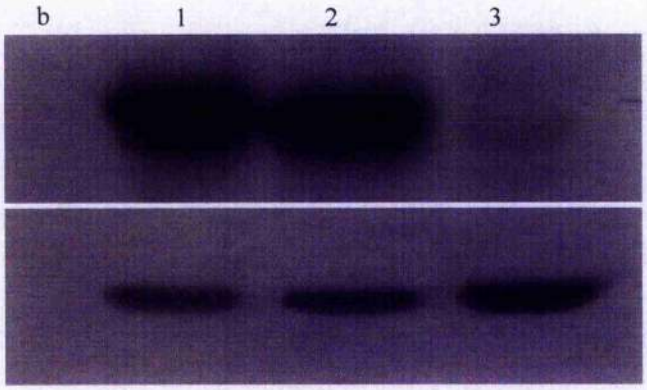
The FACS results above showed that there was no difference in the amount of total MHC I between control and cells expressing HPV-16 E5, suggesting that HPV-16 E5 in contrast to BPV-4 E5 does not interfere with MHC I heavy chain synthesis. To confirm this observation, immunoblotting analysis was performed in control P815- HLA-A2 and P815-HLA-E and in cells expressing HPV-16E5 by using mAb HC10 or mAb MEM-E/02 which specifically detect HLA-A and B heavy chain or HLA-E heavy chain respectively as described in section 2.2.47. We determined that HPV-16 E5 had no influence on HLA-A2 heavy chain (figure 25a). There was no significant difference in HLA-A2 heavy chain between control cells and cells expressing HPV-16 E5 as shown in lane 1 and 3 (mAbHC-10 did not cross react with mouse MHC I). Likewise, there was no significant difference between control and E5 cells in their expression of HLA-E heavy chain (figure 25b) lane 1 and 2. The results confirm that HPV-16 E5 does not affect the expression of HLA-A2 and HLA-E heavy chains.

Figure 25. HPV-16E5 does not affect HLA-A2 heavy chain or HLA-E heavy chain. Immunoblotting analysis was performed in control P815- HLA-A2 and P815-HLA-E and in cells expressing HPV-16E5 by using mAb HC10 or mAb MEM-E/02 which specifically detect HLA-A and B heavy chain or HLA-E heavy chain respectively. a) Detection of HLA-A2 heavy chain in control cells (lane 1) and in cells expressing 16E5 (lane 3), lane 2 indicates that mAb HC-10 does not cross react with mouse MHC I. b) Detection of HLA-E heavy chain in control cells (lane 1) and in cells expressing 16E5 (lane 2).

Figure 25 HPV-16E5 does not down-regulate expression of HLA heavy chains



1- P815HLA-A2 pcDNA
2- P815
3- P815HLA-A2 pc16E5



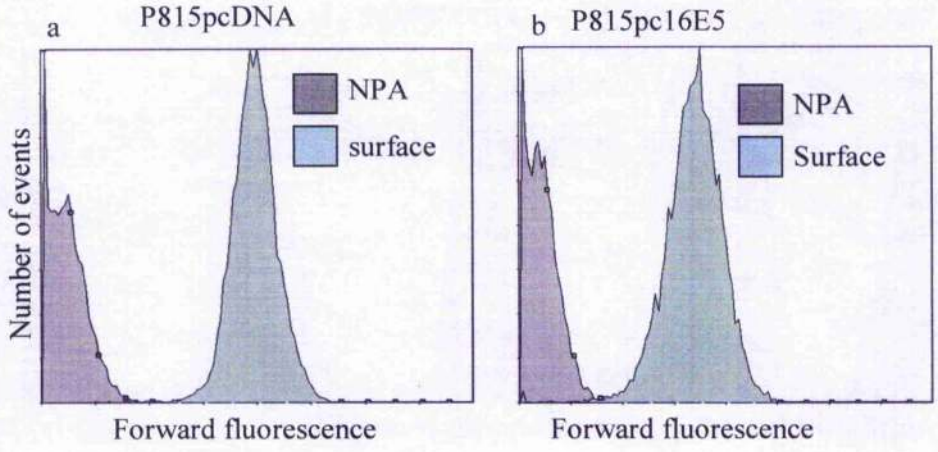
1- P815HLA-E pcDNA
2- P815HLA-E pc16E5
3- P815

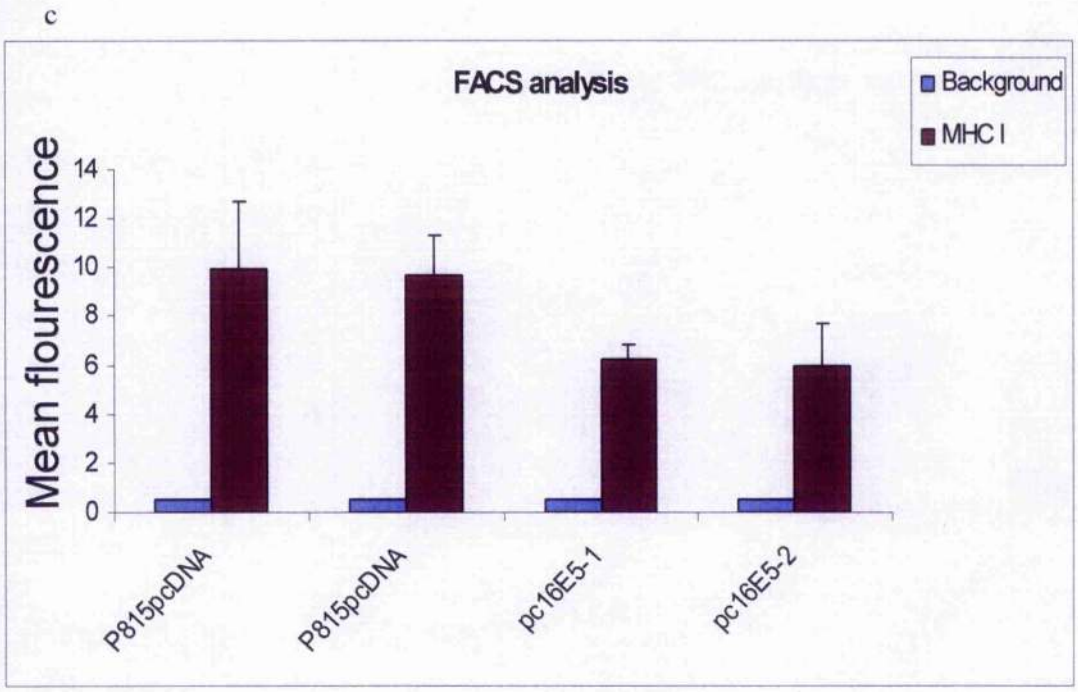
3.5.2.6 Detection of mouse H2D in P815 cells expressing HPV-16E5 by FACS

It has been shown that BPV-4E5 down-regulates the surface expression of mouse H_2-L^L in mouse fibroblast NIH3T3 (Ashrafi et al 2002). To determine the MHC I haplotype expressed in P815 cells, FACS experiment was performed by staining both parental P815 cells and NIH3T3 cells with either mAb anti-H2L or mAb anti-H2D. We found that H_2-D^d, is the most common MHC I haplotype expressed by P815 cells. To investigate whether HPV-16E5 like BPV-4E5 causes down-regulation of mouse MHC I in P815 cells, P815-pcDNA or P815-16E5 was incubated mAb anti-H2D, analysed for levels of both surface H2D. The results showed that there was no fluorescent shift from parental P815 cells or P815-pcDNA cells stained with secondary antibody only. In control cells, P815-pcDNA, there were a considerable fluorescence shift for the surface expression of H2D (figure 26a). In contrast, in P815 expressing 16E5, there was a reduction in fluorescence shift for surface H2D compared with control cells (figure 26b). To quantify the level of surface H2D in E5 cells, at least four experiments were performed and the mean of forward fluorescence shift was calculated. The results showed that P815 expressing HPV-16E5 had approximately the third lower surface H2D than control cells (figure 26c).

Figure 26. Down-regulation of mouse H2D MHC I by HPV-16 E5. P815- pcDNA cells or P815 cells expressing HPV-16 E5 were stained with mAb anti-H2D and analysed for expression of surface H2D by FACS. Example of flow cytometry profiles of surface H2D in P815 control cells (a) or cells expressing E5 (b). c) The mean forward fluorescence with standard deviation calculated from the flow cytometry profiles of at least four independent experiments performed on two cell lines.

Figure 26 HPV-16E5 down-regulates surface expression of mouse MHC I



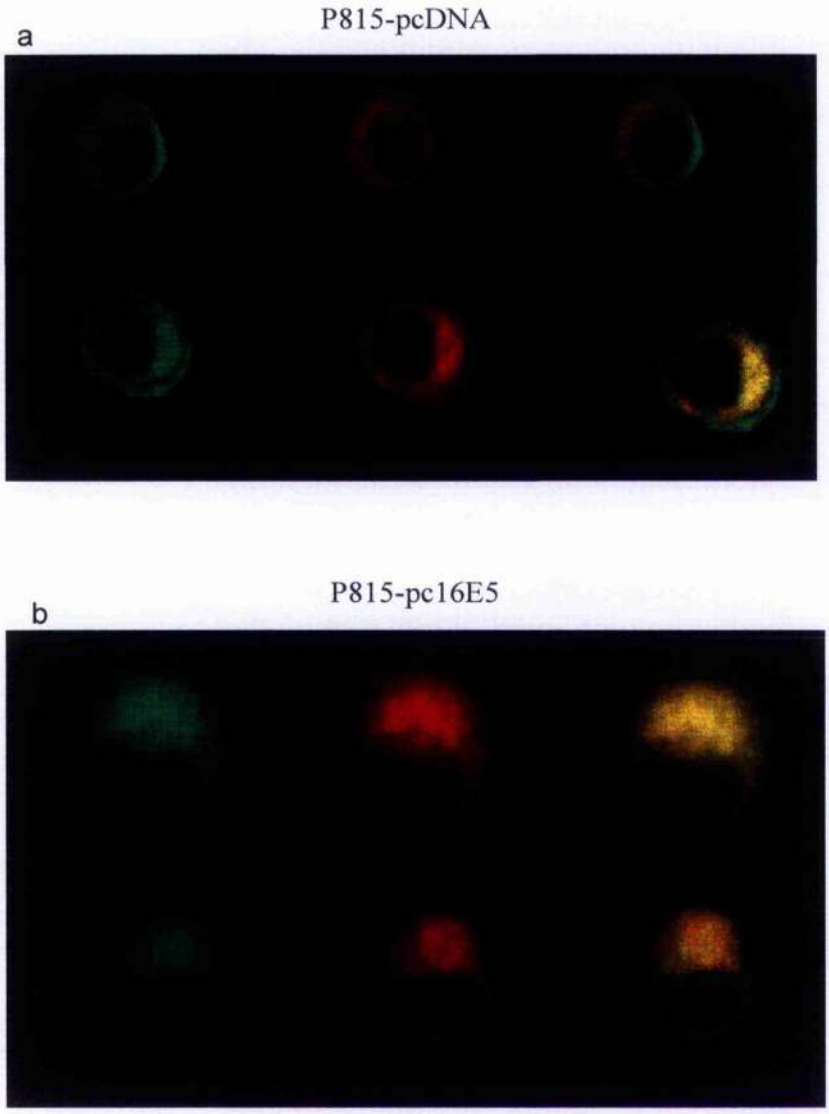


3.5.2.6.1 Detection of mouse H2D by immunofluorescence

In NIH3T3-4E5 cells, E5 retains the H2L in the Golgi apparatus (Ashrafi et al 2002). We wondered whether the reduction in surface H2D in P815 cells was also due to the retention of the complex in the Golgi. To investigate the cellular localization of H2D, we performed two-colour confocal immunofluorescence in two P815 cell lines. The cell pellet was resuspended in fixing solution for 10 min, washed twice in PBS and seeded in poly-L-lysine-pre-treated eight-well slide at 2x 10^4 cells/well following the immunofluorescence staining protocol. P815-pcDNA or P815-pc16E5 cells were stained with mAb anti-H2D and a FITC-conjugated secondary antibody, and the Golgi apparatus was detected with Bodipy Ceramide-T R. In pcDNA cells, H2D was detected both on the cell surface and in the Golgi apparatus (figure 27a) whereas in cells expressing HPV-16E5, H2D was only detected in the Golgi apparatus (figure 27b). The result shows that HPV-16E5 retains H2D in the Golgi apparatus and prevents its transport to the cell surface.

Figure 27. HPV-16E5 induces retention of H2D in Golgi apparatus in P815 cells. Two-colour confocal immunofluorescence. P815-pcDNA cells or cells expressing HPV-16 E5 were stained with mAb anti-H2D and a FITC-conjugated secondary antibody, and the Golgi apparatus was detected with Bodipy Ceramide-Texas Red. The merge between FITC and Texas Red fluorescent signals (orange) was achieved using the Leica TCS SP2 accompanying software. a) The cellular localization of H2D in control cells. b) The cellular localization of H2D in cells expressing E5. Cells at the top of panel (a) and at the bottom of panel (b) were captured at x400; cells at the bottom of panel (a) and at the top of panel (b) were captured at x600.

Figure 27 Cellular localization of mouse MHC I in P815 cells expressing E5

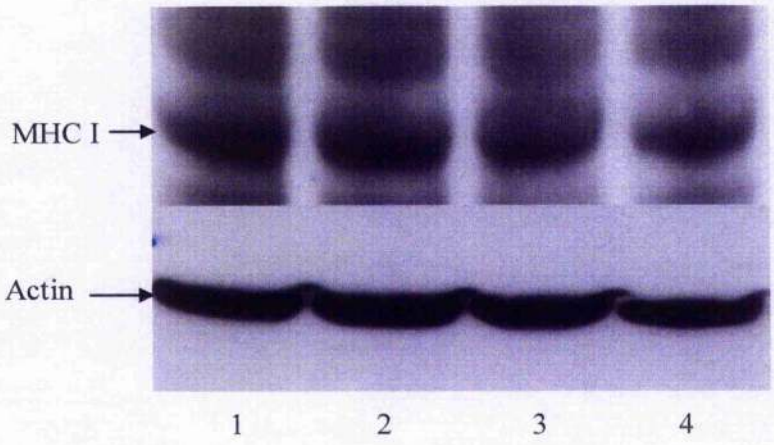


3.5.6.2 Expression of H2D heavy chain is not inhibited in P815 cells expressing HPV-16E5

To investigate whether HPV-16E5 affects expression of H2D heavy chain, immunoblotting analysis was performed in control P815-pcDNA and cells expressing HPV-16E5 by using mAb anti-mouse H2D as described in section 2.2.47. The results showed that there was no significant difference in the expression of H2D heavy chain between control and cells expressing 16E5 (figure 27c). We conclude that HPV-16E5 like BPV-4E5 in mouse fibroblasts does not down-regulate expression of mouse MHC I heavy chain.

Figure 27c. HPV-16E5 does not affect H2D heavy chain, immunoblotting analysis was performed in control P815 and cells expressing HPV-16E5 by using mAb anti-H2D for detection of H2D heavy chain and mAb AB-1, specific for β-actin. Detection of H2D heavy chain in control cells (lane 1,2) and in cells expressing 16E5 (lane 3,4).

Figure 27c HPV-16E5 does not down-regulate mouse MHC I heavy chain



3.6 HPV-16 E5 protein down regulates human MHC class I (HLA) molecules in cervical intraepithelial neoplasia (CIN).

3.6.1 Introduction

Cancer of the cervix is a very common malignant disease in women worldwide. In many developing countries, and in terms of mortality, cervical cancer is second only to breast cancer. Cervical intraepithelial neoplasia (CIN) is essentially a precursor to invasive cervical cancer, and is a condition characterized by new growth (dysplasia) in the normal epithelium of the cervix. Risk of progression of CIN is associated with persistence of high risk type HPV infection (Nobbenhuis et al 1999). While 60% of infected women with high risk HPV will develop CIN within 2-4 years of acquisition of virus, invasive cervical cancer only occurs in a small subset of cases. It has been estimated that the interval from the detection of CIN 1 to the development of cervical cancer is 13 years (van Oortmarssen et al 1992). HPV-16 is the etiologic agent of most cervical cancer and encodes three transforming oncogenes E5, E6, and E7. During viral infection, E5 is believed to play a role in the early stages of neoplastic transformation, with E5 mRNA and protein detected in low-grade CIN (Kell et al 1994; Stoler et al 1992). The expression pattern of the E5 protein is correlated with abnormal cervical cytology and can be detected at all stages of development of cervical carcinomas (Biswas et al 1997).

E5 is a highly hydrophobic protein and is found in the membrane compartment of cells. Reports have shown that the E5 protein of HPV-16 contributes to cell transformation by increasing the half life of the EGF receptors, which in turn results in transcription of its downstream factors (Straight et al 1993). It has been shown that transcription of c-fos and c-jun is induced by the E5 protein.

Complexes of c-fos/c-jun constitute the AP1 transcription factor which induces upregulation of virus transcription, thus increasing the expression of viral transforming proteins and of cellular cell cycle proteins (Bouvard et al 1994; Chen et al 1996). Moreover, HPV-16 E5 in human keratinocytes has been shown to reduce gap junction communication and may render cells insensitive to normal growth regulation signals (Oelze et al 1995). The integration of HPV DNA into the human genome has been generally accepted as a characteristic of malignant lesions which results in the disruption of E5 expression. However, recent reports have identified that HPV-16 DNA is present in episomal, integrated, and episomal/integrated forms in early cervical cancer (Choo et al 1987; Cullen et al 1991; Matsukura et al 1989; Pirami et al 1997), also HPV-16 DNA has been detected in an episomal form in one case of cervical carcinoma (Durst et al 1985). In addition to its role in the initial stages of cervical cancer, detection of E5 throughout the whole epithelium in high grade CIN lesions (Chang et al 2001) provides indirect evidence that E5 plays an important role in the development and maintenance of CIN III. Infection with HPV predisposes to cervical cancer in a multistage tumorigenic process. The host immune response plays a role in the pathogenesis of HPV-associated neoplasia: increased incidence of cervical neoplasia is observed among immunosuppressed patients (Ozsaran et al 1999; Seshadri et al 2001). The inhibition of immune response of the host against tumour is due to disorders of number and function of immune cells, altered expression of membrane proteins, including MHC molecules. Papillomavirus has evolved a mechanism to avoid immune recognition by inducing down-regulation of MHC class I by E5 proteins (Ashrafi et al 2002; O'Brien and Campo 2002). Thus persistence of lesions has been attributed, at least in part, to a disruption of

presentation of viral peptides to cytotoxic T cells as a result of interference with the MHC class I pathway by E5 protein. Down regulation of HLA class I antigens is a well-known feature of premalignant and malignant cervical epithelium. Immunologic surveillance of HPV-associated lesions is performed by T cells, which are activated when foreign (antigenic) proteins are presented to the T-cell receptor by HLA class I proteins. Loss of HLA class I is associated with severity of CIN lesions and HLA is significantly down-regulated in more aggressive lesions (Torres et al 1993). Occurrence of CIN is accompanied by down-regulation of specific HLA class I alleles (Wang et al 2001). It has been reported that up regulation of selected HLA class I allele expression, induced by IFN-gamma, correlates with the resolution of cervical intraepithelial lesions and high-risk HPV DNA clearance *in vivo* (Sikorski et al 2004). *In vitro*, HPV-16 E5 has been shown to induce down regulation of HLA class I in immortalized human keratinocytes (Ashrafi et al 2005). No correlation between E5 and HLA class I expression in cervical lesions has been made (Hilders et al 1994; Hilders et al 1995). The aim of this part of the study is to investigate whether, as in the case of bovine papillomas, expression of E5 correlates with absence of HLA class I.

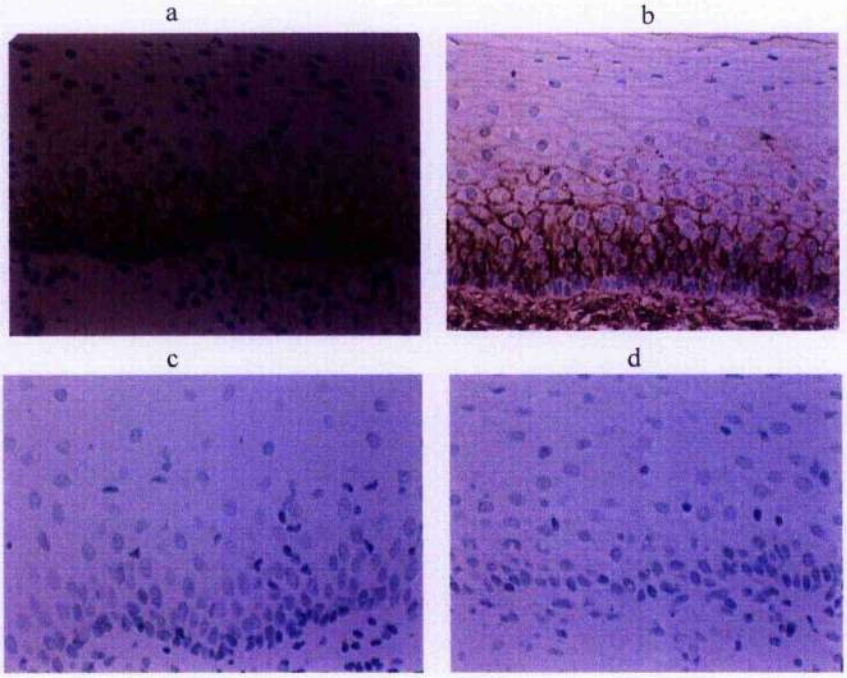
3.6.2 Expression of HLA in normal cervix

HLA-ABC antigens are expressed constitutively by most normal nucleated cells (Daar et al 1984), but may be lost during transformation to the neoplastic state. To evaluate the expression of HLA class I in the normal cervix, 10 paraffin blocks of normal cervix from hysterectomies and biopsies taken for reasons unrelated to HPV infection were obtained from Dr David Millan, NHS, Glasgow. Sections were deparaffinised, re-hydrated through a graded series of alcohol and

endogenous peroxidase was inhibited in methanol containing 1.5% hydrogen peroxide. After antigen retrieval, any background staining that could result by incubation with secondary antibody was avoided by applying normal rabbit serum. Sections were then incubated with mAb HC10, which detects HLA-A, B and C heavy chains. Sections were incubated with biotin-labeled rabbit anti-mouse secondary antibody. Signal was amplified by staining sections with avidin-biotin-peroxidase. HLA class I staining was visualised in brown colour by applying DAB to sections. HLA class I staining was firstly evaluated microscopically and images were captured by using images analysis software (Zeiss KS300). There was strong HLA class I staining in basal and suprabasal layers with a clear staining pattern, fainter staining was detected in the upper layers (figure 28a,b). HLA staining was also observed in derma (stroma, lymphocytes). The specificity of HLA class I staining was verified by incubation of sections without mAb HC10 (figure 28c,d).

Figure 28. Detection of HLA class I on paraffin sections from normal cervix. a and b) Representative examples of immunostaining for HLA class I by using mAb HC-10; c and d) Verification of the specificity of HLA class I staining, normal sections were stained with secondary antibody only. x200 magnification.

Figure 28 Sections of normal cervix incubated with HC10

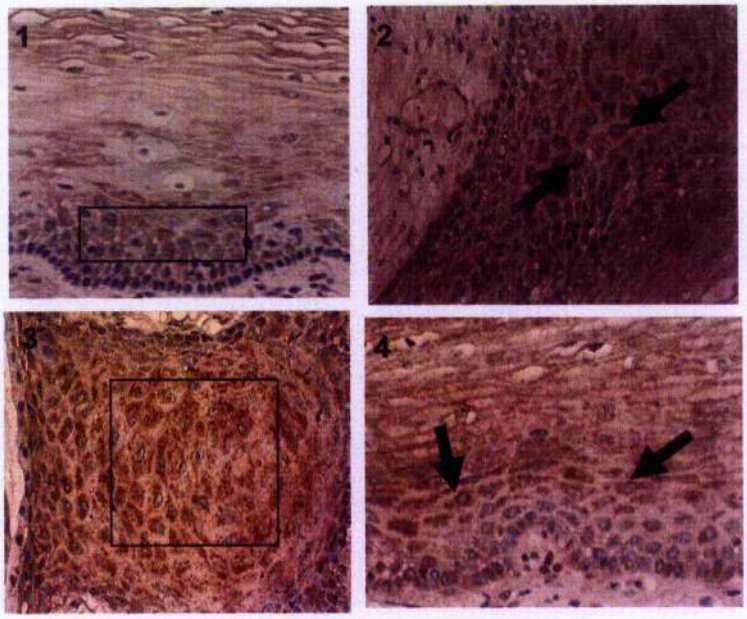


3.6.3 Expression of HPV-16E5 in CIN

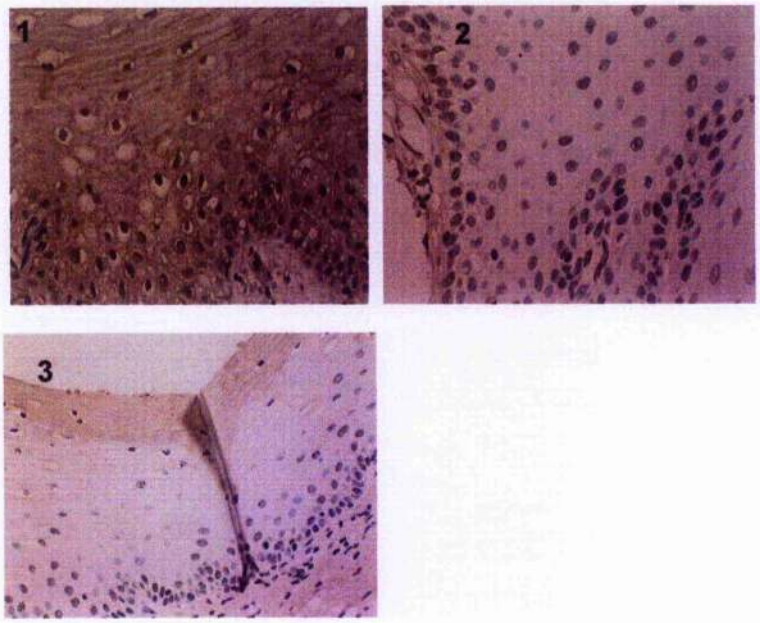
To determine the expression pattern of HPV-16 E5, immunostaining analysis was performed on paraffin sections representing 10 samples of CIN I using antisera against the N and C terminal halves of HPV-16 E5 (Hwang et al 1995). We have chosen CIN I to investigate the expression of HPV-16E5 as E5 is most likely to be expressed in the early stages of infection, although E5 has been shown to be expressed in CIN II and CIN III which are more advanced stage of infection (Chang et al 2001). Sections were incubated with unlabeled swine serum, and then incubated with E5 antisera at ratio 1:1. Sections were incubated with biotin-labeled swine anti-rabbit immunoglobulin and biotinolated HRP streptavidin complex following immunohistochemistry protocol. Brown staining of cytoplasm indicated E5 expression which was detected mainly in the suprabasal (figure 29a 1,2) and granular layers of the epithelium (figure 29a 3,4). The staining of keratinized layers is mostly background as shown by the staining of normal section with E5 antisera (figure 29b-1). The specificity of E5 staining was verified by incubating CIN sections without E5 antisera (figure 29b-2,3). We conclude that the cytoplasmic detection of E5 in the suprabasal layer confirms that PV E5 proteins are expressed in the deep layers (Anderson et al 1997) where E5 is sufficient to play a role in avoiding immune recognition by down-regulation of MHC I. Detection of E5 in the upper layers confirms its role in the productive stages of viral life cycle (Fehrmann et al 2003; Genther et al 2003).

Figure 29. Expression of HPV-16E5 in CIN I. Representative CIN I and normal sections were stained with E5 anti-serum. a) Panel of CIN sections show detection of E5 mainly in suprabasal as indicated by rectangular (a-1) and by arrows (a-2) while (a 3,4) show detection of E5 mainly in granular layers as indicated by square (a-3) and by arrows (a-4). b) Verification E5 specificity of staining, (1) normal section was stained with E5 anti-serum. (2,3) CIN sections were stained with secondary antibody only. (a-1,2,3 and 4 and b-1,2) x200 magnification, b-3 x100 magnification.

Figure 29 a) Detection of HPV-16E5



b) Verification of E5 staining



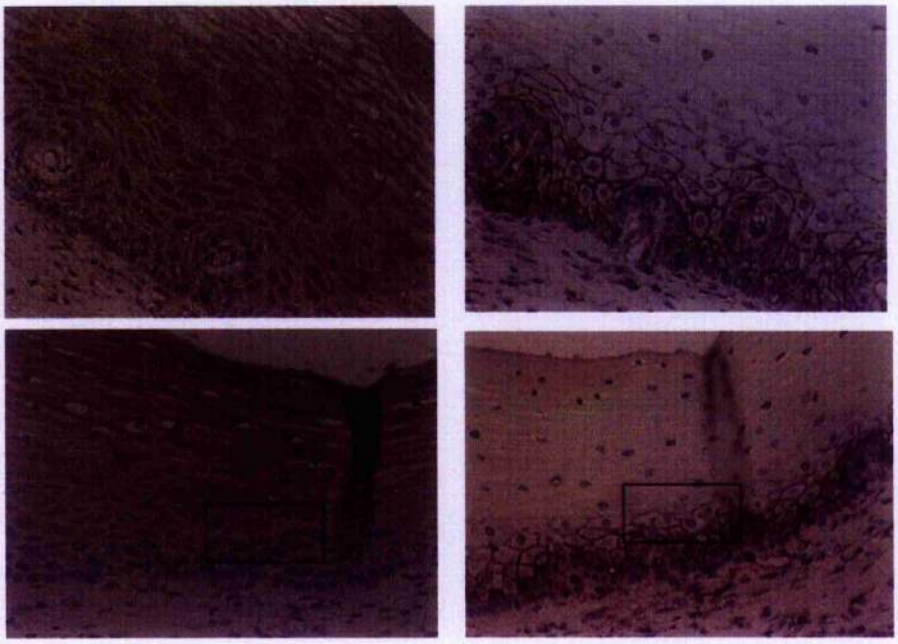
3.6.4 Expression of HLA in CIN

To determine whether expression of HPV-16 E5 in CIN is associated with down regulation of HLA class I, serial sections of CIN were stained either with mAb HC-10 for MHC I detection or with anti-E5 antisera as above. Slides were microscopically examined by looking at E5 staining patterns in particular areas and matching them with the same areas in the section stained for HLA class I. Two staining patterns were observed: in 2 out of 10 samples, E5 and HLA class I were detectable in the same cells (figure 30a); in 6 out of 10 samples, no HLA class I was detected in cells where E5 expression was observed and vice versa E5 positive cells did not express HLA class I (figure 30b). Occasionally, in 2 out of 10 samples, a mixed pattern was observed (figure 30c). Theses results suggest that also in CIN 16E5 is responsible for down-regulation of HLA class I.

Figure 30. Expression of HLA class I in different CIN I lesions. Representative CIN I sections were stained with mAb HC10 for HLA detection or with E5 antiserum. a) Panel of CIN show detection of HLA class I and HPV-16E5 in the same CIN I cells; b) HLA class I was not detected in E5 positive cells; c) mixed staining pattern of HLA class I in the same CIN section. (a, c and lower 4 panels of b) x200 magnification, (upper b) x100 magnification.

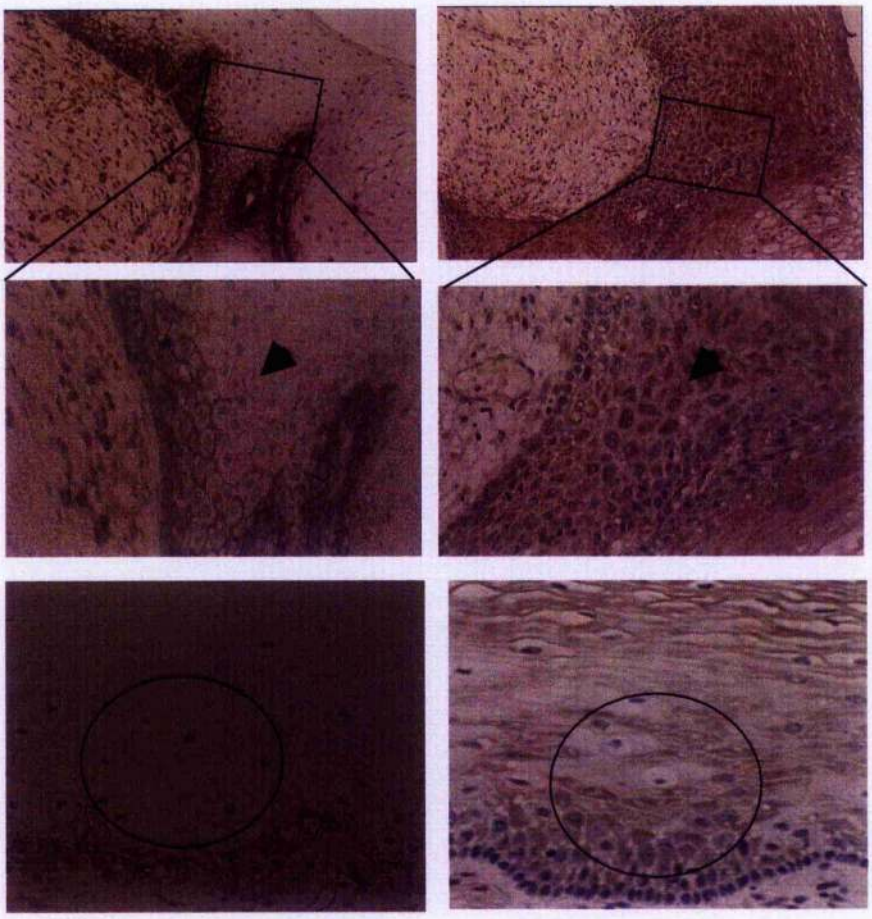
Figure 30 Expression of HLA class I in CIN sections

a) Detection of E5 and HLA in the same cells

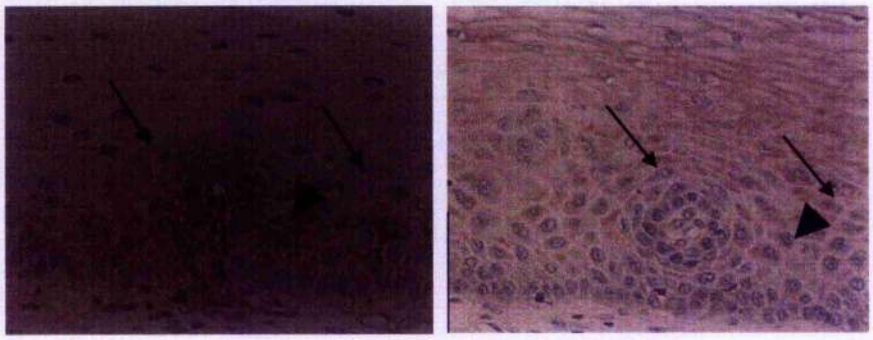


E5 HLA

b) No HLA in cells expressing HPV-16E5



c) Mixed pattern of a and b



HLA E5

3.6.5 The proliferation state in CIN

The Ki-67 is nuclear antigen expressed in proliferating cells throughout the cell cycle except during the G0 and early G1 phases. The Ki67 antigen is predominantly localized in the nucleus and has an important role in the maintenance and regulation of the cell division. To evaluate the proliferation state of CIN cells, serial sections representing 10 samples from CIN I, as well as 10 samples from normal cervical epithelium were either incubated with mouse monoclonal MIB-1 antibody which recognizes nuclei of proliferating cells or with anti- E5 antisera. Sections stained for Ki67 were incubated with rabbit anti-mouse secondary antibody whereas sections stained for E5 were incubated with swine anti-rabbit as described in section 2.2.9. Signal was amplified by staining section with avidin-biotin-peroxidase. Staining was visualised by applying DAB to sections. The slides were examined microscopically; the Ki67 positive nuclei and E5 positive cells were identified and correlated. Images from selected areas were captured by using images analysis software (Zeiss KS300).

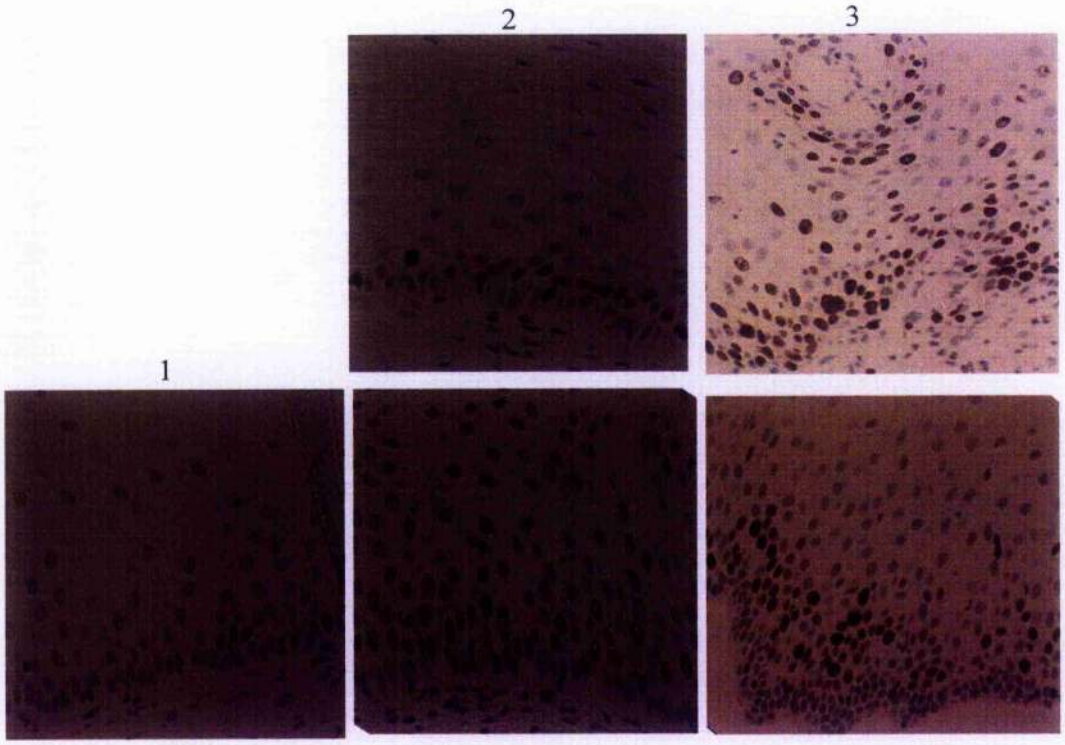
In normal epithelium, only a few nuclei of basal and suprabasal cells were stained with monoclonal antibody MIB-1 against the nuclear antigen Ki67. In contrast, in most CIN sections (8 out of 10 samples), the intensity of MIB-1 staining was strong and the expression of Ki67 was detected throughout the lower third of epithelium with exception for few CIN sections in which the expression of Ki67 was detected in suprabasal cells with lower number of positive cells (figure 31a). Most basal cells were negative both in normal cervical epithelium and in CIN I. The results show that in some cases the expression of Ki67 was associated with expression of HPV-16E5 throughout the lower third of epithelium (figure 31b). Although HPV-16E5 is the minor transforming protein compared with E6 and E7,

according to *in vitro* data which showed that E5 contributes to the cell cycle by activating the positive regulators and inhibiting the negative regulator of cell cycle, in our experiments, no correlation was made between expression of E6 or E7 and Ki67 expression. This result is in agreement with, Fehrmann *et al*, (2003), suggesting that a potential role of E5 in cell transformation is to increase cyclin A and B expression and subsequently overcome the differentiation-induced cell cycle arrest. Also, expression of Ki67 throughout the lower third suggests that these lesions have the potential to progress to CIN II / CIN III to invasive cancer (Kruse et al 2004). Restriction of Ki67 expression to the suprabasal layer in some CIN sections would suggest that the cells proliferate slowly and that these lesions are unlikely to progress.

Figure 31. Expression of Ki67 in CIN I. a) Representative CIN I sections show two different pattern of Ki67 expression; in the basal and suprabasal layers, and throughout the lower third of epithelium. b) Serial CIN I sections were stained with E5 anti-serum or mAb MIB-1, Ki67 was detected throughout the lower third of the epithelium, in some cells, Ki67 expression was accompanied by expression of HPV-16E5. (a and b) x200 magnification.

Figure 31

a) Different expression patterns of Ki67 in CIN I

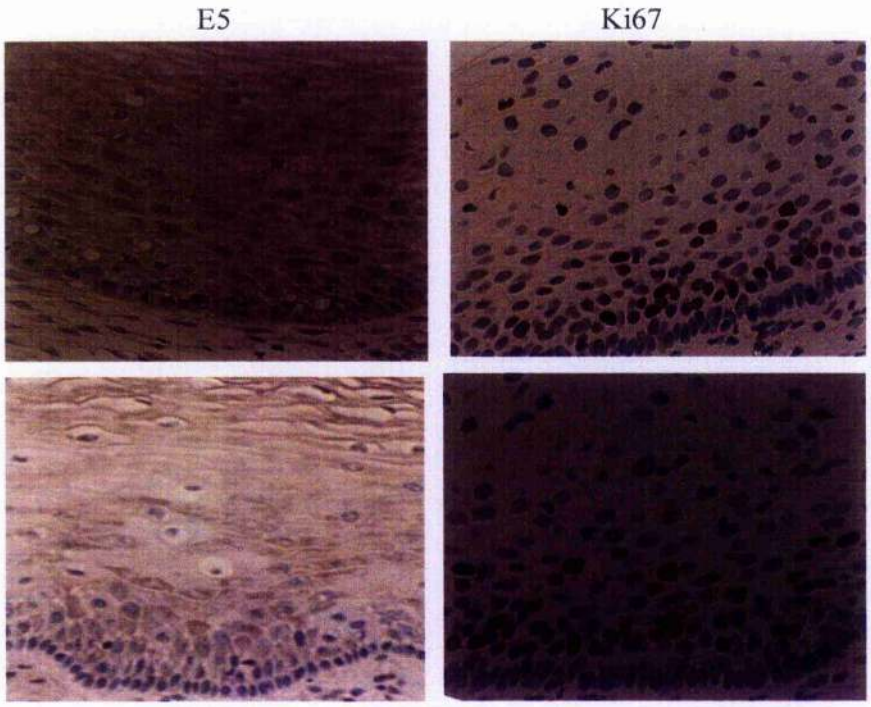


Normal

Deep layers of Lower third

Whole lower third of epithelium

b) Co-expression of HPV-16E5 and Ki67 in CIN I



3.7 HPV-16 E5 protein down-regulates HLA in raft cultures

Organtypic epithelial raft cultures are tissue culture systems that allow keratinocyte monolayer to stratify and achieve full differentiation via culturing of the cells on collagen gels at the air-liquid interface. Histologically, the raft cultures have similar structure and morphology to the intact epithelia. We cannot rule out that down-regulation of HLA class I in CIN is only due to expression of E5 since E6 and E7 are also expressed in these lesions and down-regulation of HLA class I is in part due to expression of by E7 (Georgopoulos et al 2000). Furthermore, the viral load of high-risk HPV DNA was strongly correlated with CIN II and CIN III when compared with women with only CIN I or normal cervix (Santos et al 2003). Hence we investigated the expression of HPV-16 E5 and its impact on changing patterns of MHC class I expression in epithelial layers using artificial epidermis that mimics the normal one in stratification, differentiation and the diversity of MHC class I patterns among epithelial layers. The immortalized human HaCaT cells were stably transfected with two different plasmid pcDNA or PL2 carrying HPV-16E5 cDNA as described (Ashrafi et al 2005). The transfected HaCaT cells were cultured on collagen gels for 10-14 days and the stratified cells were harvested and embedded on paraffin blocks. The culture of these cells was made by Dr Sheila Graham, IBLS, Glasgow University. To examine the effects of E5 on epithelial morphology and differentiation, sections of raft from HaCaT parental, pcDNA and cells expressing E5 were stained with haematoxylin and eosin. Control cells differentiated, with nuclear staining predominantly localized to cells in the basal layer (figure 32a). However, the cultures were not "fully normal", as occasionally invasion of the keratinocytes into the collagen was observed (figure 32b). HaCaT cells expressing E5 showed an altered differentiation pattern, with a

thickening of the basal layer and nuclear staining throughout all layers (figure 32c). Also there was abnormal distribution in the keratin; extensive keratinisation was localized in the lower epithelium instead of the superficial layer (figure 32d). These observations confirm that E5 stimulates cellular proliferation via its effects on cell cycle regulators and epidermal growth factor receptors. This hyperkeratinization resembles the one observed in HPV-16E5 transgenic mice (Genther Williams et al 2005).

To determine down-regulation of HLA class I and expression of HPV-16 E5 in artificial stratified epithelium, serial raft sections from HaCaT parental, pcDNA and cells expressing E5 were either stained with anti-E5 antisera, HC10 for detection of classical HLA class I or mAb MEM-E/02, specific for HLA-E as described in section 2.2.9. In HaCaT control cells, both classical and non classical HLA class I were detected in most epithelial layers with clear staining pattern (figure 33a and b). In contrast, HaCaT cells expressing pc16E5 (figure 33c), did not express classical HLA class I (figure 33d), while they retained expression of HLA-E (figure 33e). These results confirm that HPV-16E5 down-regulates classical HLA class I, but does not down-regulate non classical HLA-E.

It has to be noted that the analysis of the relationship between E5 and HLA class I in CIN and in raft culture is preliminary and requires more experimentation.

Figure 32. Morphology and differentiation pattern of HaCaT cells in raft culture sections stained with haematoxylin and eosin. (a and b) Differentiation of parental HaCaT rafts. (c and d) Alteration of the differentiation pattern of HaCaT cells expressing HPV-16E5. (a and b) x200 magnification, (c and d) x400 magnification.

Figure 32

HPV-16E5 causes alteration in morphology and differentiation pattern of HaCaT cells

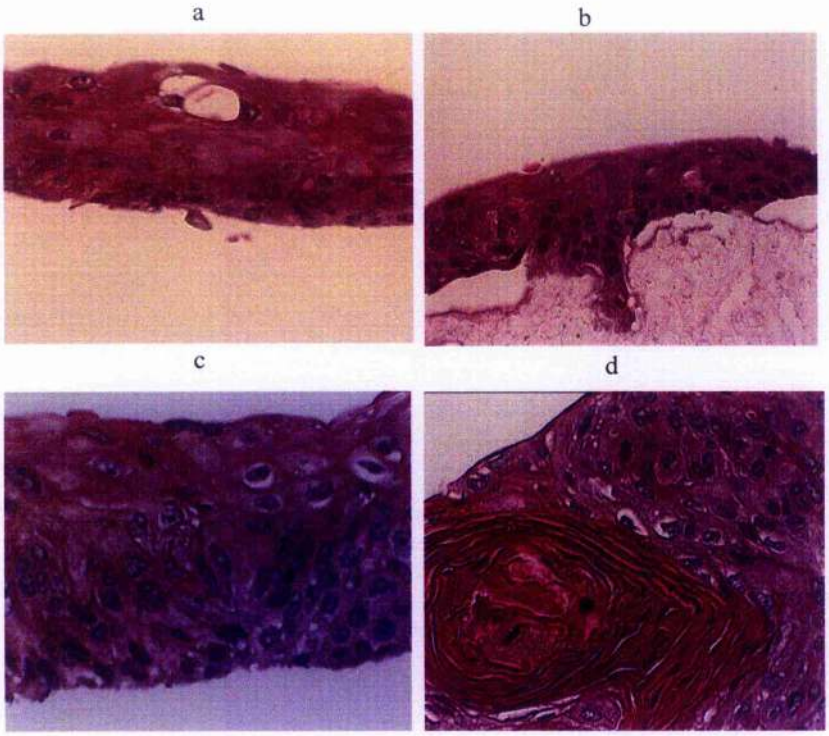


Figure 33. Expression of HLA class I in raft culture of HaCaT cells. a) Expression of classical HLA class I in parental HaCaT cells. b) Expression of non classical HLA-E in parental HaCaT cells. c) Expression of HPV-16E5 in HaCaT cells. d) Expression of classical HLA class I in HaCaT cells expressing HPV-16E5. e) Expression of HLA-E in HaCaT cells expressing E5. (a) x400 magnification, (b,c,d and e) x200 magnification

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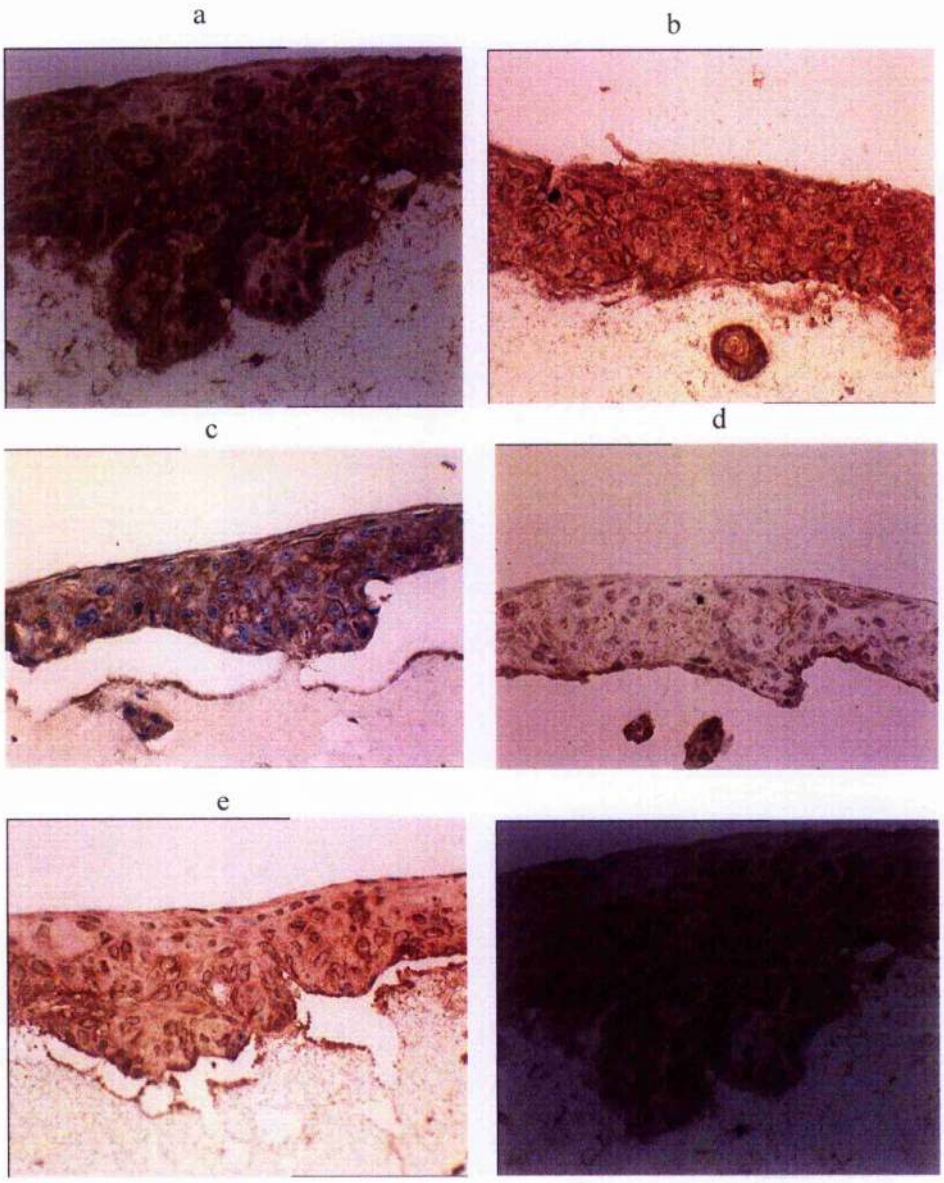
Figure 33 HPV-16E5 down-regulates classical HLA class I but does not affect HLA-E



Chapter Four

Discussion

4 Discussion

4.1 Introduction

Viruses are totally dependent upon host biosynthetic machinery for replicating their genomes and establishing infection. These processes are associated with the production of antigenic proteins that make the infected cell susceptible to immune surveillance which ultimately results in elimination of viral infection. However, viruses have developed mechanisms to escape cellular immune recognition by encoding proteins that interfere with antigen presentation to immune cells. In general, recognition and subsequent elimination of virally-infected cells involves three major immune effector mechanisms: Ab-dependent lysis, MHC class I-restricted CTL-mediated lysis and NK cell-mediated lysis. These mechanisms are influenced by cytokines, which function as chemical messengers between cells (Diamond 2003). One mechanism by which viruses evade clearance by the host immune response is to inhibit CTL recognition by down-regulation of MHC-I expression on the surface of infected cells. Cells are always under surveillance by CTLs, which continuously examine somatic cell surfaces for MHC class I molecules presenting non-self peptides derived from viral antigens. MHC class I molecules are particularly attractive targets for immune evasion by viruses, because decreasing expression and/or antigen presentation by MHC class I can attenuate CD8 T-cell-mediated recognition of infected cells (Yang and Wilson 1995). Both self and foreign antigens are degraded in the cytosol to peptides which are translocated into the endoplasmic reticulum where they are loaded onto MHC I molecules and are transported via the Golgi apparatus to the cell surface (Yewdell and Bennink 1992).

Papillomaviruses infect cutanous and mucosal epithelium, causing benign lesions which persist for several months to one year before regression by cell-mediated immunity. However, occasionally some lesions, particularly those caused by BPV-4, can persist and transform to squamous cell carcinoma in the gastrointestinal tract of cattle. This is particularly evident in cattle grazing on bracken fern which contains mutagens including quercetin; these act as dietary factors for malignant conversion. Also, in individuals who are HPV-16 infected, CIN lesions can progress and develop into cervical cancer, particularly in presence of immunosuppressive diseases such as HIV infection in which failure of immune recognition is accompanied by malignant conversion (O'Brien and Campo 2003).

The ability of papillomaviruses to persist for significant periods of time has been linked to the nature of infection which indirectly subverts host immune responses to the infected cells by minimising the exposure of virus to immune cells. Papillomaviruses can potentially subvert the immune response by interference with antigen presentation to CTLs by down-regulation of MHC I (Ashrafi et al 2002). Papillomavirus E5 protein is mainly localized to Golgi apparatus and endoplasmic reticulum and causes impairment of the MHC I trafficking to the cell surface. Recently, it has been found that BPV-4 E5 protein physically interacts with the heavy chain of MHC class I and irreversibly retains the MHC complex in the Golgi apparatus (Marchetti et al 2006). Like other oncogenic viruses, which interfere with several steps of MHC class I pathway, BPV-4 E5 proteins have wide effect on MHC class I; they inhibit MHC class I gene transcription, protein stability and trafficking of MHC class I molecules to cell surface. These viral strategies used to prevent peptide/MHC-I presentation at the surface of infected cells may be

protective against CTL recognition and destruction. However, they leave the infected cells susceptible to lysis by another arm of the host's immune system, the NK cells, because of the reduced opportunity to engage NK cell inhibitor receptors (Biassoni et al 2001). However, it has been found that the inhibitory receptors recognize MHC leader sequence peptide presented by the non classical MHC class I molecules such as HLA-E (Braud et al 1998a). These molecules are able to deliver negative signals to NK cells through immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic domain, which act to recruit tyrosine phosphatases, thereby cutting positive signal pathways (Eissmann et al 2005; Watzl 2003; Lanier 1998).

4.2 BPV-4E5 induces down-regulation of bovine classical MHC I but does not affect non classical MHC I in P815 cells

Because there is no monoclonal antibody available which exclusively recognizes non-classical class I molecules, to study the relationship of BPV-4 E5 with classical and non-classical MHC I, we separately expressed a classical, *N*01301*, and a non-classical, *N*50001*, allele in mouse P815 cells and subsequently introduced E5 in these cells. This proved to be a powerful system which has confirmed that E5 down-regulates classical MHC class I, while it does not interfere with the biosynthetic pathway of this non-classical MHC class I.

4.2.1 BPV-4E5 and classical MHC class I

It has been shown in bovine PalF cells (Marchetti et al., 2006), that E5 down-regulates classical MHC class I at more than one level. However, in PalF cells, E5 was co-expressed with E6, E7 and activated *ras* (Pennie et al., 1993), leaving open

the possibility, albeit remote, that down-regulation of MHC class I was due to a functional interaction between the viral and cellular oncoproteins. In P815 cells, we have found that E5 profoundly inhibits expression of both surface and total classical MHC class I in the absence of other viral or cellular oncoproteins (figure 13). It has been shown that BPV-4E5 down-regulates mouse MHC class I in NIH3T3 cells and bovine MHC class I in PalF cells (Ashrafi et al 2002). We have shown that BPV-4E5 down-regulates bovine classical N*01301 MHC class I in P815 cells (figure 13) and human classical B2705 MHC class I expressed in 4E5-PalF cells (figure 17c). Our observations demonstrated that down-regulation of MHC class I by BPV-4E5 is not species specific. In P815 cells, unlike PalF cells, the Golgi structure was not affected in cells expressing E5. It has been suggested that the Golgi fragmentation is due to the combined action of BPV-4E5 and other viral proteins and activated *ras*. However, it is still to be determined whether Golgi fragmentation is due to the combined action only in bovine cells (tissue specific) or can be induced in P815 cells by co-transfection of P815 cells with BPV-4E5, E6, E7 and activated *ras*.

4.2.2 BPV-4E5 and non-classical MHC class I

We investigated the effect of E5 on expression of non classical MHC class I. N*50001 is bovine non-classical MHC class I, it differs from classical N*01301 MHC class I; N*50001 lacks an intracytoplasmic domain. We found that in marked contrast to its inhibitory effect on classical MHC class I, E5 does not affect non-classical MHC class I. Non-classical MHC class I reach the cell surface (figure 14) and non-classical N*50001 HC is expressed to the same levels as in control cells (figure 18). It has been shown that HPV-16E5 does not affect the

localization of HLA-E on the cell surface of HaCaT cells (Ashrafi et al 2005). Thus, it is possible that the presence of non-classical MHC class I on the cell surface of E5 expressing cells would protect these from killing by NK cells which become activated as result of lacking classical MHC class I on the cell surface.

4.2.3 How does BPV-4E5 down-regulate N*01301?

Immunological surveillance of viral infection is performed by T cells, which are activated when foreign antigenic proteins are presented to the T-cell receptors by class I MHC molecules. The ability of MHC class I molecules to sample the intracellular environment and present antigens to CTLs poses a great threat to viruses and other intracellular pathogens. In order to circumvent this problem, many viruses have evolved strategies to interfere with the MHC class I antigen presentation pathway. It is now clear that all individual steps in MHC class I pathway are targeted by viral proteins that modulate the host immune response (Wagner et al 2002). Redirecting MHC class I heavy chains into the cytosol for degradation by subversion of Sec61 is one of the most important steps in the MHC I pathway targeted by viral proteins. Sec61 is an important translocon which consists of complex proteins associated with the translocation of nascent polypeptides into the cisternal space of the endoplasmic reticulum from the cytosol and re-directs transport of mis-folded proteins back into the cytosol where they are rapidly degraded by the proteasome (Romisch 1999). This retrograde transport system is commandeered by US2 and US11 proteins of HCMV which route newly synthesised MHC I HC to Sec61 for rapid dislocation into the cytosol and degradation by proteasome (Wiertz et al 1996). Also it has been reported that the p12 protein of HTLV-1 binds to the HLA class I heavy chain thus preventing its

association with β_2M, and reroutes MHC I HC into the cytosol for proteasomal degradation (Johnson et al 2000). It has also been shown that HIV Nef protein binds to MHC I heavy chain, creating a binding site for the adaptor protein AP-1 which in turn leads to the recruitment of MHC I into the lysosomes for degradation (Roeth et al 2004). Moreover, Nef protein may decrease heavy chain gene transcription via its activation of c-jun (Fackler et al 1999) as a complex of c-jun/c-fos acts as a negative trans-acting factor that down regulates MHC I gene expression (Howcroft et al 1993). Papillomaviruses, like other viruses, have been implicated in interference with many steps of MHC class I pathway. It has been shown that BPV-4 E5 inhibits transcription of the MHC class I heavy chain gene and transport of the complex to the cell surface (Ashrafi, 2002; Marchetti et al., 2006). In P815 cells, in addition to blocking MHC class I in the Golgi apparatus and preventing its transport to the cell surface, E5 induces the degradation of N*01301 HC. In control cells, the half-life of the HC is approximately 20 hours, whereas in the presence of E5 its half-life is dramatically reduced to less than 5 hours. We do not yet know how E5 causes MHC class I heavy chain degradation. HIV Nef protein shares similarity with E5 protein and as mentioned before Nef re-directs HC to the lysosomes for degradation through the simultaneous interaction with HC and with the AP-1 complex. As suggested by Marchetti et al., (2006), it is thus possible that E5 also interacts with both HC and AP-1 inducing lysosomal degradation of HC, but this remains to be elucidated.

Transcription of the bovine N*01301 HC gene cannot be analysed in P815 cells: E5 inhibits transcription from the homologous promoter of the HC gene (Marchetti et al., 2006), while in P815 cells the N*01301 HC gene is under the control of the CMV IE promoter.

4.2.4 Down-regulation of MHC I is mediated by the C-terminal domain of BPV-4E5

Previous studies have shown that the E5 oncoprotein of BPV-4 interacts physically with 16k subunit c, leading to loss of gap junctional intercellular communication. It has been shown that both the hydrophilic C-terminal tail and the residue at position 17 in the hydrophobic domain are crucial for the transforming activity of E5 protein (O'Brien et al 1999). It is reasonable to assume that a profound effect on MHC class I transport is a consequence of the interaction between E5 and MHC I HC. We investigated whether down-regulation of MHC I can be attributed to E5/MHC I HC interaction and whether the C-terminal domain or position 17 is responsible for E5/HC interaction and down-regulation of MHC I. We have shown that E5 and HC proteins co-precipitate *in vitro* and *in vivo* (Marchetti et al., 2006) and the E5 mutant N17Y binds HC but does not inhibit MHC class I expression. The E5T mutant which lacks the C-terminal domain, as reported by Ashrafi et al., (2002), does not inhibit the expression of MHC class I or its transport to the cell surface. Also, E5T does not interact with HC and the interaction between E5 and HC is prevented by a peptide corresponding to the C-terminus of E5 (Marchetti et al., 2006). Our observations have demonstrated that potential immune evasion through down regulation of MHC I is accompanied by cell transformation (Ashrafi, 2002; O'Brien, 2003), and that the C-terminus domain of BPV-4E5 which mediates the transforming activity of E5, is also responsible for E5/HC interaction and down- regulation of MHC I.

4.3.1 HPV-16E5 causes down-regulation of MHC class I

HLA-A, B and C encode transmembrane glycoproteins which are involved in the process of immune recognition. Class I molecules are heterodimers consisting of a polymorphic heavy chain non-covalently associated with an invariant light chain (termed β2-microglobulin) and they function by binding intracellularly processed peptides, presenting them on the cell surface to cytotoxic T lymphocytes (Williams et al 1996). Individual subunits of glycoproteins are synthesized in the rough endoplasmic reticulum (RER), where they fold and may undergo modifications such as disulfide bond formation and glycosylation (Hurtley and Helenius 1989). Transport of peptides from the cytosol to the ER is mediated by a transporter associated with antigen processing (TAP). In the ER, the assembly of newly synthesized MHC class I heavy chain (HC) molecules with β_2-microglobulin (β_2-m) and peptide is assisted by transient interactions with a number of ER-resident chaperones. Following peptide association, class I molecules dissociate from TAP and the stable MHC class I/β_2-m /peptide trimer transits from the ER *via* the trans Golgi to the cell surface for presentation to CTL.

Localization of papillomavirus E5 proteins in Golgi and other cytoplasmic compartments causes interference with Golgi functions (Schapiro et al 2000). Since processing of MHC I takes place in these compartments and its transport to the cell surface takes place through the Golgi, it is to be expected that translocation of MHC I complex to the cell surface would be disrupted in E5 cells. Previous studies have shown that HPV-16E5 down-regulates HLA-A and B but not HLA-C/E in HaCaT cells (Ashrafi et al 2005). We confirmed this observation by investigating the effects of E5 on expression of HLA-A2 in mouse mastocytoma P815 cells. We have shown that HPV-16E5 down-regulates HLA-A2 on the cell

surface, reducing the level of surface HLA-A2 to approximately half of that in the control cells (21). Furthermore, we found that HLA-A2 was retained in the GA (figure 23). The effect of HPV-16E5 on MHC I is similar to effects of BPV-4E5 in retaining MHC I within the Golgi and reducing its levels on the cell surface, but does not affect HLA-A2 heavy chain (figure 25). Prevention of MHC I transport to the cell surface is a biological characteristic shared by papillomaviruses E5. It has also been shown that HPV-2a E5 and HPV-83E5 can inhibit MHC I transport to the cell surface (Cartin and Alonso 2003; Ashrafi et al 2006). Thus, down-regulation of surface MHC class I seems a characteristic displayed by many papillomavirus E5 proteins.

4.3.2 HPV-16E5 does not down-regulate HLA-E

The cell surface expression of non classical HLA-E inhibits NK cell-mediated lysis by interacting with inhibitory NK receptors. There are specifically two types of receptors for self MHC class I molecules to inhibit NK cells, including killer cell immunoglobulin-like receptors (KIR) that predominantly recognize classical MHC class I, and the lectin-like hetrodimer CD94/NKG2 receptors that recognize HLA-E (Braud et al 1998a). The absence of classical MHC class I on the cell surface of the virus-infected cells causes these to be susceptible to killing by NK cells unless HLA-E molecules are presented on the surface. It has been shown that HPV-16E5 does not interfere with expression of HLA-E/C on the surface of HaCaT cells, but it could not be determined whether the localization of MHC I on the surface was HLA-E or HLA-C as the mAb DT9 cross-reacts with both of them. For this reason we have used the powerful system of P815 cells to introduce HLA-E cDNA into cells expressing 16E5. The expression of HLA-E was analyzed using mAb DT9.

Our observations have confirmed that HPV-16E5 does not down-regulate the expression of HLA-E (figure 22). We conclude that HPV-16 E5 down-regulates HLA-A2 by retaining it in the Golgi apparatus, but it does not affect HLA-E. Additionally, HPV-16E5 down-regulates the surface expression of mouse H2D MHC class I by one-third. HPV-16E5 induces down-regulation of MHC class I in HaCaT, PalF, and NIH3T3 cells (Ashrafi et al., 2005). Therefore, down-regulation of MHC class I by HPV-16E5 like BPV-4E5 is not species specific.

4.4.1 Expression of BPV-4 E5 induces cellular proliferation in papillomas

The cell cycle in mammalian cells is regulated by the coordinated activity of a family of cyclin-dependent kinases (CDKs). Cyclin A promotes both G1/S and G2/M transitions. Cyclin A is also required for DNA synthesis and once transported into the nucleus, it forms a complex with CDK, which is required for entry into mitosis (Pagano et al 1992). Previous studies have reported that BPV-4E5 expression in NIH3T3 cells promotes transcriptional activation of the cyclin A promoter and increases endogenous protein levels, thus allowing proliferation in the absence of mitogens (O'Brien and Campo 1998). In the present study, we have also shown that expression of the proliferation marker Ki67 is associated with expression of E5 in differentiated keratinocytes, while in normal epithelium Ki67 staining is found only in the proliferative basal cells. In agreement with *in vitro* studies we conclude that BPV-4 E5 causes alteration in the cell cycle and forces post-mitotic cells to re-enter the cycle and proliferate.

4.4.2 Expression of HPV-16 E5 induces cellular proliferation in CIN I

HPV-16 E5, along with the more transforming E6 and E7 proteins of the virus, has been found to be oncogenic. E5 transactivates viral genes via the activation of AP-1 and thereby increases viral E6/E7 gene expression (Chen et al 1996a). E5 may also play role in cell transformation by regulating the expression of other cellular and viral genes. It has also been demonstrated that E5 proteins of HPV-11 and -16 repress the expression of tumour suppressor gene p21 and impair the control of the cell cycle checkpoint, and this might be one of the mechanisms by which E5 stimulates cell proliferation (Tsao et al 1996). In our present study we investigated the Ki67 immunostaining as an index of cellular proliferation in CIN I and correlation between the Ki67 and HPV-16 E5 expression was analysed. We have shown that there are two staining pattern of Ki67 in CIN I; in approximately 80% of CIN I samples, the Ki67 expression was detected throughout the lower third of epithelium including the basal layer with more intensity of positive nuclei (figure 31a-3,b), in 20% of samples, Ki67 was detected in the basal and suprabasal layers with lower numbers of positive cells and no staining in intermediate layers and, in some cases, no significant difference between normal and CIN epithelium (figure 31a-1,2). Our results are in agreement with Kruse et al, 2004, who proposed that lesions in which Ki67 is expressed throughout the lower third of epithelium are likely to progress, while lesions in which Ki67 is restricted to basal and suprabasal layers are unlikely to progress. Unlike in bovine papillomas, expression of Ki67 is only partially associated with E5 in some CIN cells. This may be due to the fact that HPV-16E5 is a minor transforming protein while E6 and E7 are the major transforming proteins and progression to malignancy is due to E6 and E7, which disrupt the pathways of p53 and of p105 Rb.

4.5 Down-regulation of MHC class I in papillomas and CIN and rafts

We have shown that BPV-4E5 and HPV-16E5 down-regulate bovine classical MHC class I and human classical HLA-A2 respectively by using the powerful system of P815 cells. We extended our observations to investigate the effect of E5 on MHC class I in naturally infected cells on paraffin sections of papillomas and CIN by using immunohistochemistry.

4.5.1 BPV-4 E5 protein down-regulate MHC class I in bovine papillomas

We investigated the relationship between the expression of BPV-4 E5 and expression of MHC class I in naturally infected papilloma cells immunohistochemically by using E5 antisera and mAb IL-A88 for detection of MHC class I. We have found that E5 was detected exclusively in the cytoplasm of epidermal cells, from the basal and parabasal layers to the spinous and squamous layers. Cells that expressed E5 did not express MHC class I. We conclude that in bovine papillomas, expression of E5 is clearly not compatible with expression of MHC class I. Our observations are in agreement with previous studies (Anderson et al 1997; Burnett et al 1992) and confirm the hypothesis of the functions of E5 protein in natural infection; expression of E5 protein in the basal layer supports its role in immune evasion by down regulation of MHC class I in those cells that are accessible to immune surveillance. Expression of E5 protein in differentiated layers supports its role in viral replication and packaging of the viral genome (Fehrmann et al 2003). In addition, expression of E5 protein in the intermediate layers supports its role in the maintenance of the transformed state of papillomas.

4.5.2 Down-regulation of MHC I is due to expression of E5 not E7

Previous studies have shown that the E7 proteins of HPV-16 and -11 are implicated in the down-regulation of MHC I either through inhibition of the transcriptional promoter of the MHC I heavy chain (Georgopoulos et al 2000) or indirectly through inhibition of TAP, the transporter associated with peptide (Vambutas et al 2000) respectively. In our present study, we have shown that in cells that expressed E5 and did not express E7, or expressed it at levels below detection, there was little or no MHC I. On the contrary, cells that expressed BPV-4 E7, but not E5, still had detectable MHC I. We conclude that although cooperation between BPV-4 E5 and E7 is necessary to achieve full transformation, down-regulation of MHC I is only induced in the presence of E5 and E7 is not responsible for down-regulation of MHC I.

4.5.3 HPV-16E5 protein down-regulates MHC class I in CIN I

It has been shown that HPV-16E5 down-regulates HLA-A/B but it does not down-regulate HLA-C/E in cultured HaCaT cells (Ashrafi et al 2005). We confirm that HPV-16E5 induces down-regulation of HLA-A2 by using the powerful system of P815 cells. Down-regulation of MHC class I has been observed in premalignant lesions and squamous carcinomas of the uterine cervix (Chil et al 2003). In our present study, we extended our observations to investigate the relationship between expression of HPV-16E5 and down-regulation of MHC class I in paraffin sections of CIN I. We have found that in approximately 60 % of CIN I, expression of HPV-16E5 is not compatible with expression of MHC class I and in 40 % of samples, E5 and MHC class I are co-expressed. We conclude that although some E5 cells do not express MHC class I, down-regulation of MHC class I in CIN I, unlike in

bovine papillomas, needs further analysis. It has been shown that E7 proteins of HPV-11 and HPV-16 are in part responsible for down-regulation of MHC class I. We did not investigate the relationship between expression of E7 and expression of MHC class I. Therefore the reduction or absence of MHC class I expression in CIN I cells may be due to expression of HPV-16E7. Absence of MHC class I was predominantly observed in cells of higher layers, while the cells in very deep layers still expressed MHC class I. We hypothesize that a reduction in MHC I is not required in deeper layers as E6 and E7 expression is very limited; the low expression of E6 and E7 within these cells is insufficient to raise a host immune response. Because no host immune response is raised, there is no requirement for immune evasion mechanisms to be generated by the virus. In the middle and upper layers, E6 and E7 are expressed at much greater concentrations requiring that the virus block antigenic presentation via manipulation of MHC I. More investigation is required to validate this hypothesis.

4.5.4 HPV-16E5 protein down-regulates MHC class I in raft culture

Because we did not investigate expression of HPV-16E7 in CIN, we cannot state that down-regulation of MHC class I in CIN I is only due to expression of HPV-16E5. In the present study, we firstly investigated the impact of E5 on the morphology and differentiation of HaCaT cells in raft sections. It has been shown that expression of HPV-16 in raft cultures of primary keratinocytes induces alteration in the differentiation pattern of these cells (McCance et al 1988). Moreover, raft cultures of cell lines derived from cervical intraepithelial neoplasia exhibited abnormal differentiation patterns similar to those of cervical intraepithelial neoplasia *in vivo* (Rader et al 1990). It has been shown that

expression of HPV-31 E5 in rafts of human foreskin keratinocytes induces an altered differentiation pattern, with a thickening of the basal layer and nuclear staining throughout all layers (Fehrmann et al 2003). It has been shown that expression of HPV-11 DNA in organotypic raft culture induces thickness in the epithelium and extensive keratinisation (Fang et al 2006). We have found that expression of HPV-16 E5 induces hyperkeratinization and a highly proliferative morphology of HaCaT rafts compared with control rafts. We investigated expression of HLA-A2 and HLA-E in HaCaT rafts expressing HPV-16 E5. As expected, given that all cells express E5, no cells express classical HLA-A (figure 33d), but they do express HLA-E (figure 33e). These observations confirm that HPV-16E5 down-regulates classical MHC class I but does not down-regulate HLA-E even during stratification and abnormal differentiation.

4.6 Future work

Future work should be focused on the effects of BPV-4E5 on MHC class I. It has been shown that HIV Nef protein binds to MHC I heavy chain, creating a binding site for AP-1 which in turn leads to the recruitment of MHC I into the lysosomes for degradation (Roeth et al 2004). The mechanisms by which BPV-4E5 causes degradation of MHC class I heavy chain should be investigated. It has been found that BPV-4E5 still interacts with MHC heavy chain lacking the C terminal tail of HC, suggesting that there are other domains in HC that are essential for the physical interaction with E5. It should be determined which amino acids in the HC are essential for interaction with E5.

It has been shown that expression of BPV-4E5 increases the pH of the Golgi, which is thought to be a result of its binding to 16k subunit c (Schapiro et al 2000).

The alkalization of the Golgi interferes with processing and transporting of proteins including the MHC complex. It has been shown that BPV-4E5 physically interacts via its C terminal domain with MHC class I heavy chain and this interaction is associated with the down-regulation of MHC complex. This suggests that BPV-4E5 reduces the presentation of MHC class I on the cell surface both by interacting with 16K subunit c and by binding to MHC heavy chain. It should be determined whether the physical interactions of BPV-4E5 with the MHC heavy chain and with 16K subunit c can take place concurrently.

We have shown that expression of HPV-16E5 down-regulates expression of HLA class I in some CIN I cells. However, other CIN cells that express E5 still have detectable HLA class I. Unlike bovine papilloma cells in which expression of BPV-4E5 is not compatible with expression of MHC class I, down-regulation of HLA class I by HPV-16E5 in CIN I is still not clear and more investigations of CIN I and CIN II sections are required to elucidate the relationship between expression of E5 and HLA class I.

It has been suggested that lack of surface expression of classical MHC class I in infected epithelial cells of bovine papillomas and CIN expressing BPV-4E5 and HPV-16E5 respectively would allow evasion of destruction by cytotoxic T lymphocyte and therefore establishment of viral infection. The presence of non classical MHC class I would inhibit destruction of these cells by NK cells. It is important to clarify this point by applying CTL and NK killing assays on E5-expressing cells.

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