CD4 T lymphocyte responses to human papillomavirus type 16.

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A thesis submitted for the degree of

Doctor of Philosophy

June 1999

University College London
Abstract

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Human papillomavirus type 16 (HPV16) is the commonest of the oncogenic papillomaviruses and is the main cause of two thirds of cervical carcinoma worldwide. There is good reason to believe that HPV infection is controlled by a cellular immune response. The demonstration and characterisation of human CD4 positive T lymphocytes primed by HPV16 would be very useful for the development and evaluation of vaccines to prevent or attenuate HPV16 infection. To date it has not proved possible to detect such lymphocytes using conventional means.

A novel and simple method is described of detecting antigen-specific primed CD4 T cells in the peripheral blood. This has been used to detect cells primed to HPV16 and to influenza nucleoprotein but could be used to detect responses to other antigens.

Using this method, responses to HPV16 have been detected in three women with documented CIN (these responses were chiefly to E6 and E7) and in two young uncharacterised control individuals (to L1 alone). In three individuals it was possible to repeat the assays and the responses were found to be transient.

Using a completely different approach, evidence is presented that DNA immunisation in the hind legs of mice generates a response specific for HPV16 L1 in the para aortic lymph nodes and that cells taken from these nodes can be restimulated in vitro by HPV16 L1 protein.

A set of overlapping 25mer peptides representing HPV16 E7 and L1 could not restimulate human or murine responses although at least two of them were immunogenic in mice. Reasons for this are discussed.
Acknowledgements

I am very grateful to Professor Peter Beverley for his continual support, encouragement and friendship.

I would also like to thank everyone at the tumour immunology group for help of various kinds. In particular Diana Wallace, Julian Hickling, Maria Dahl, Harry White, Lindsey Goff, Pip O'Brien and Chris Hughes for practical assistance and Hal Drakesmith for always having an answer.

Meloo Preston and Elizabeth Mansell helped enormously in recruitment of patients and Linda Ho and George Terry with HPV16 detection by PCR.

Finally I thank my wife Andrea who sacrificed much to allow me to complete this work and who provided unstinting support when the going became difficult.
Note on changes to 1999 version

This thesis was first submitted in August 1997. The alterations for the 1999 version include a clarification and reorganisation of the methods sections, the inclusion of more detailed results of proliferation assays (appendix) and expansion and updating of the account of the molecular biology of the virus. However the main thrust of the thesis - the immune response to HPV16 - has not been altered. There have been some developments in this area including further accounts of CTL and proliferative responses to HPV in women (de Gruijl et al. 1999, Gill et al. 1998, Kadish et al. 1997, Nimako et al. 1997), further claims of HLA associations with disease (Bontkes et al. 1998, Helland et al. 1998) and an influential account of the coincidence of HPV-associated cervical cancer with a polymorphism of p53 (Storey 1998). None of these publications has necessitated any important change in the arguments or discussion of the immunology of the virus. Further work has continued on vaccination strategies (for instance Steller et al. 1998) but as yet it remains unclear whether vaccination will be useful in the prevention or treatment of HPV-associated disease.


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<tr>
<td>APC</td>
<td>antigen presenting cell</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
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<tr>
<td>BPV</td>
<td>bovine papillomavirus</td>
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<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
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<tr>
<td>CFA</td>
<td>complete Freund's adjuvant</td>
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<tr>
<td>CIN</td>
<td>cervical intraepithelial neoplasia</td>
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<tr>
<td>COPV</td>
<td>canine oral papillomavirus</td>
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<tr>
<td>CRPV</td>
<td>cottontail rabbit papillomavirus</td>
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<tr>
<td>CTL</td>
<td>cytotoxic lymphocyte</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's modification of Eagle's medium</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DTH</td>
<td>delayed type hypersensitivity</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid (disodium salt)</td>
</tr>
<tr>
<td>EEV</td>
<td>extracellular enveloped virus (vaccinia)</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorter</td>
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<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>GST</td>
<td>glutathione S transferase</td>
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<td>HBV</td>
<td>hepatitis B virus</td>
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<td>HIV</td>
<td>human immunodeficiency virus</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HLA</td>
<td>human leucocyte antigens</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>HPV</td>
<td>human papillomavirus</td>
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<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
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<tr>
<td>IFA</td>
<td>incomplete Freund's adjuvant</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>ICRF</td>
<td>Imperial Cancer Research Fund</td>
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<tr>
<td>IMDM</td>
<td>Iscove's modified Dulbecco's medium</td>
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<tr>
<td>IMV</td>
<td>intracellular mature virion (vaccinia)</td>
</tr>
<tr>
<td>KLH</td>
<td>keyhole limpet hemocyanin</td>
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<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>MPA</td>
<td>mycophenolic acid</td>
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<td>NK</td>
<td>natural killer</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<td>ORF</td>
<td>open reading frame</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units (vaccinia)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>PHA</td>
<td>phyto haemagglutinin</td>
</tr>
<tr>
<td>PPD</td>
<td>purified protein derivative of tuberculin</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylene diamine</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>VLP</td>
<td>virus-like particle</td>
</tr>
<tr>
<td>VVCd1</td>
<td>recombinant vaccinia with murine cyclin D1</td>
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<tr>
<td>VVE6</td>
<td>recombinant vaccinia with HPV16 E6</td>
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<tr>
<td>VVE7</td>
<td>recombinant vaccinia with HPV16 E7</td>
</tr>
<tr>
<td>VVL1</td>
<td>recombinant vaccinia with HPV16 L1</td>
</tr>
<tr>
<td>VVNP</td>
<td>recombinant vaccinia with influenza nucleoprotein</td>
</tr>
<tr>
<td>VVWT</td>
<td>wild type vaccinia virus</td>
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Chapter 1

Introduction
Papillomavirus biology

Papillomaviruses are small DNA viruses which replicate only within epithelial cells. They produce a characteristic lesion known as a papilloma or wart. The infectious particle is an icosahedral capsid of diameter 52-55 nm which encloses a single, circular, double stranded DNA molecule of about 8000 bp (Baker et al. 1991). Although papillomaviruses are classified together with polyomaviruses as belonging to the papovavirus family they are quite distinct. Polyomaviruses, which include the human pathogens known as JC and BK, have a smaller capsid (about 45 nm) and a smaller genome (about 5000 bp) as well as a different genetic organisation (Howley 1996).

Papillomaviruses have been identified infecting many different mammals, some birds and even turtles but although these viruses are all very similar in genetic organisation and content their tissue tropism is so strict that infection across species, although reported, is very unusual (Koller and Olson 1972). Even within a single species infection can usually only occur in a particular type of epithelium so, for instance, HPV16 requires human anal or genital epithelium to establish a productive infection. A subgroup of papillomaviruses cause fibropapillomas which contain proliferating dermal fibroblasts as well as squamous epithelial cells. This subgroup includes the much studied viruses BPV1 and BPV2 (Howley 1996).

Classification

Papillomaviruses are classified firstly according to the species they infect and secondly according to the degree of homology of the DNA sequence. By convention a virus isolate was designated a new type if there was less than 50% homology with known types. This was determined by DNA:DNA hybridisation and S1 nuclease digestion (Coggin and zur Hausen 1978). More recently it has been decided to compare DNA sequence in the L1 E6 and E7 ORFs. A new type should have less
Each of the identified HPV types has a predilection for a cutaneous or mucosal surface. At least 25 HPV types infect the genital tract and these can be divided into two groups: those associated with genital warts - the commonest examples of which are HPV types 6 and 11 and those associated with high grade intra-epithelial lesions and invasive cancer of which HPV 16 is the commonest and types 18, 31, 33, and 35 are also represented. The HPV types associated with cervical cancer are often referred to as "high risk" or "oncogenic" types.
Molecular biology of papillomaviruses

The genomes of more than 22 human papillomaviruses and more than 7 non-human papillomaviruses have been completely sequenced (Howley 1996). In spite of this a great deal remains to be learned about the biology of these viruses, not least because they remain very difficult to propagate in vitro. In particular the determinants of their strict tissue tropism and the molecular mechanisms underlying the tightly controlled programme of transcription and replication are still largely obscure.

Genome structure and organisation

All papillomavirus genomes are very similar in organisation. The genes are encoded on one strand of DNA only, this strand contains eight to ten ORFs which are designated "early" or "late" by analogy with the organisation of SV40 virus and depending on their location in the genome (Baker 1993) (figure 1.1). The "early" region genes (E1 to E7) facilitate and regulate viral replication, gene transcription and cellular transformation. The "late" region genes (L1 and L2) encode the viral capsid proteins. There is a long stretch of DNA in all papillomaviruses which contains no ORF, this is known as the upstream regulatory region and contains the origin of replication as well as cis-acting sequences which regulate gene expression.

Papillomavirus replication cycle

The tropism of papillomaviruses for differentiating epithelium has made their life cycle very difficult to study in the laboratory. However organotypic culture systems have been developed which allow keratinocyte differentiation (Stanley 1994a). When keratinocyte cell lines which contain episomal HPV are grown in such cultures they stratify and differentiate. There is viral DNA amplification and late gene expression in the upper layer of stratified cells. When these cultures are treated with an activator
of protein kinase C there is capsid protein synthesis and virion production (Meyers et al. 1992). HPV31b DNA in the form of recircularised genomes has also been transfected into primary foreskin keratinocytes to create episomal HPV DNA artificially. Such cells added to an organotypic culture system then allow the production of viral particles in the same way (Frattini, Lim, and Laimins 1996). These systems have allowed the study of the distribution of viral transcripts in differentiating epithelia and also the way in which viral genomes are maintained as extrachromosomal elements, an essential requirement for late gene expression (Frattini, Lim, and Laimins 1996).

**Virion entry into cells**

As yet no culture system has been able to support the full life cycle and it remains difficult to study the early stages of infection. It is very likely that successful infection requires virion entry into stem cells. HPV capsid proteins L1 and L2 self-assemble *in vitro* into virus-like particles (VLPs) (Kirnbauer et al. 1992) and using these some progress has been made in studying viral entry into cells. Papillomavirus VLPs and infectious virions bind to a wide range of cell types, the absence of L2 from VLPs makes no difference in the binding properties (Roden et al. 1996). Recent evidence has suggested that α6β4 integrin is a component of a VLP "receptor". Antibody to α6β4 greatly reduces the binding of VLPs to cells. Freshly isolated keratinocytes expressing high levels of α6β4 have the *in vitro* characteristics of stem cells. The process of internalisation and uncoating is not understood but the early stages at least could involve continuing association with α6β4 (Evander 1997). The promiscuity of cell binding of PVs implies that tropism is not determined at the cell surface. Instead it could well depend on aspects of the differentiation programme of the infected epithelium, important early stages of which take place in the stem cell (Stanley 1994b). The lag period of several weeks between infection and clinical appearance of a papilloma (Oriel 1971) or detection of viral activity in animal models (Kreider and Bartlett 1981, Stoler et al. 1990) can be
explained if the infected cell has a long cycle time. Most keratinocytes in an epithelium will have fully differentiated and been shed from the surface within 3 weeks, leaving the stem cell as the only likely candidate.

The monoclonal nature of many papillomas (including CIN) suggests that the entry of a single virion into a single stem cell can be sufficient to establish infection (zur Hausen and de Villiers 1994).

Expression of viral genes

Electron micrography has shown that all PV mRNA is transcribed from the same DNA strand in the same direction. The transcripts are polycistronic and code up to four ORFs. There is a large variety of mRNAs because different promoters are used and the transcripts have variable splicing patterns (Petterson et al. 1986).

Expression of viral mRNA and protein and replication of viral DNA has been shown to be closely tied to keratinocyte differentiation both for CRPV (Kreider and Bartlett 1981) and HPV. Detailed study of DNA replication and mRNA expression has been carried out using a xenograft model with HPV11 (Stoler et al. 1990). Viral DNA was at such low levels in the basal layer that it could not be detected by in situ hybridisation (below about 50 copies per cell with the method used). The most abundant mRNA throughout encoded E4 protein. Low levels of E1, E2, E6 and E7 mRNA could be detected from the parabasal layer and higher levels of E6 and E7 were present in the more differentiated cells. It was only in the most superficial cells that high levels of viral DNA could be detected together with capsid proteins and whole virions. This pattern of gene expression and DNA replication is also true for HPV16 (Durst et al. 1992). There is barely detectable transcription in the basal layers of low grade CIN but strong signals in the upper layers.

This control of viral gene expression must have a profound influence on the way the virus is seen by the immune system.
Viral replication

It is probably accurate to view PVs as having two modes of replication. Firstly in the cells of the basal layer the DNA is maintained as a stable multi-copy plasmid with tightly controlled replication. The viral genome replicates on average once per cell cycle and is passed on to the daughter cell (Chow and Broker 1997). Secondly, in differentiated epithelial cells where cellular DNA replication has ceased there is a burst of DNA synthesis which generates viral genomes to be incorporated into infectious virions. It is perhaps not surprising that these two modes of replication seem to employ two completely different mechanisms. Examination of replicative intermediates of DNA has shown that in the non-productive stage (an environment which should be favourable for DNA replication as the cells are mitotically active) DNA replicates bi-directionally via theta structures and so requires initiation with every round of replication whereas in the productive stage when cellular DNA is not being replicated the virus seems to undergo so-called "rolling circle" replication where one initiation will allow multiple copies of DNA (Flores and Lambert 1997).

Function of open reading frames.

E1 and E2

E1 and E2 are involved in the control of both viral replication and transcription, they have been studied mainly in BPV1. In order to replicate in a host cell the virus must override cellular control mechanisms and provide any necessary enzyme activity not available in the cell (Kuo et al. 1994). E1 and E2 provide these control functions and E1 encodes enzyme activity including DNA binding, ATP binding, ATPase, DNA unwinding and helicase activity (Yang et al. 1993).

The E1 ORF is the largest in the genome and is well conserved, the full length BPV1 E1 protein binds specifically to the origin of replication, its affinity is increased by the binding of full length E2 to an adjacent site (Mohr et al. 1990, Ustav et al. 1993).
E2 is closely involved in the regulation of viral transcription. Full length E2 controls all the major early promoters, however the transactivation function of E2 is not essential for the production of virions in an organotypic culture system (Stubenrauch, Colbert, and Laimins 1998).

Both E1 and E2 proteins are required for replication of HPV11 (Chiang et al. 1992). The observation that BPV1 gene products will also support HPV replication suggests that restrictions on viral DNA replication do not determine the tissue and species tropism (Chiang et al. 1992).

**E4**

Eight or more E4 proteins have been identified, they range from 10 - 34 kDa in size. The most abundant of these is the primary gene product, a 17kDa protein which is in fact a fusion protein including the N terminus of E1. The other species are derived from this by proteolytic cleavage or dimerisation or a combination of these (Doorbar et al. 1992). Mutational analysis has shown that BPV1 E4 is not needed for transformation or for DNA replication (Neary, Horwitz, and DiMaio 1987) and HPV16 E4 is not involved in capsid assembly (Zhou et al. 1991b). One function which has been suggested for it is to aid assembly or release of virions in some way. E4 protein accumulates along with the capsid proteins in the outer layers of an infected epithelium. In HPV1 at least there is an enormous accumulation in papillomas where it can contribute up to 30% of cellular protein (Doorbar et al. 1986). When HPV16 E4 was introduced into human keratinocyte cell lines using recombinant vaccinia the protein accumulated in association with cytokeratins and disrupted the intermediate filament network (Doorbar et al. 1991). However, although HPV1 E4 does associate with the cytokeratin network it does not lead to its collapse in cultured cells (Roberts et al. 1993) and differentiated epithelial cell cultures show no evidence of such collapse in the presence of E4 (Sterling, Skepper, and Stanley 1993).
**L1 and L2**

The "late" region of HPV DNA contains two ORFs which encode the two components of the viral capsid. L1 is the major component, it makes up 90% of the capsid and will self-assemble in vitro into VLPs without the need for L2 (Kimbauer et al. 1992). Such particles are less homogeneous and probably less stable than VLPs when L2 is included (Hagensee, Yaegashi, and Galloway 1993).

L1 and L2 are expressed in the cytoplasm of differentiated epithelial cells. They both have nuclear localisation sequences and are quickly transported to the nucleus. L2 seems to be responsible for guiding L1 to specific locations in the nucleus where viral assembly takes place. In addition it has the property of binding DNA without the need for sequence specificity, this has led to the suggestion that it has the role of binding the capsid to the viral genome (Sun et al. 1995).

**E5**

BPV1 E5 protein alone is able to transform some rodent cells in culture. In murine fibroblasts it seems to do this by forming a stable complex with the cellular receptor for platelet-derived growth factor (Petti and DiMaioa 1994). Although it has been found that HPV16 E5 has transforming ability in some systems (Leptak et al. 1991) it is not expressed in human cancers so is clearly not required for maintenance of the transformed state.

**E6 and E7**

These are the major transforming proteins of oncogenic papillomaviruses and are discussed in the section on transformation.
Figure 1.1

A linear depiction of the 7905bp circular genome of HPV16. The positions of the ORFs have been inferred from the complete DNA sequence (Baker 1993).
HPV16 causes human cancer

DNA from oncogenic HPV is found integrated in the genome of nearly all cervical carcinoma (Walboomers et al. 1994) and epidemiological evidence now points firmly at oncogenic HPV infection as a prerequisite for most, if not all, cervical cancer. A recent case control study using carefully controlled PCR methods has avoided many of the problems of earlier studies and has shown a strong association between detection of HPV16, 18, 31, 33 or 35 and the presence of cervical carcinoma (Munoz et al. 1992).

Oncogenic HPV infection also predisposes to other squamous carcinomas in the anogenital region. This includes carcinoma of the vagina, vulva, penis and anus (Daling and Sherman 1992). However all of these cancers are much rarer than cervical carcinoma and they occur in a much older age group, suggesting both that HPV infection must have persisted for longer and that the necessary co-factors are less common at these sites.

Some other cancers are also probably caused by HPV including squamous carcinoma of the skin - particularly in the immunosuppressed and in association with the rare condition of epidermodysplasia verruciformis (zur Hausen and de Villiers 1994).

Mechanism of cellular transformation

Apart from the epidemiological evidence the most striking confirmation that oncogenic HPV can cause cancer is the transforming activity in vitro of the E6 and E7 proteins. In order to establish a productive infection HPV must interfere with cellular mechanisms controlling proliferation. It seems likely that the potential for establishing malignancy is a by-product of this property of viral genes.

The first observations of transformation followed the introduction of whole viral DNA into cultured rodent cells (Yasumoto et al. 1986). Subsequently, the use of
individual genes demonstrated that the E7 protein alone was sometimes sufficient although the addition of E6 has a synergistic effect. The more stringent requirements for maintaining immortalisation in cultured human foreskin cells requires the continued presence of both E6 and E7 (Hawley-Nelson et al. 1989). E5 is not needed in spite of its central role in transformation by BPV1 and its weak transforming activity in some human cells. E6 and E7 proteins from non-oncogenic HPV are far less efficient at transformation in these assays (Storey et al. 1988).

HPV16 E7 protein is a small, zinc binding phosphoprotein of 98 amino acids. It has sequence and probably functional similarities with the E1A protein from adenovirus and large T from SV 40, two other DNA viruses with transforming properties. The function which these proteins have in common is in binding to cellular proteins which control progress through the cell cycle. All three viral proteins bind to Rb and p107. E7 and adenoviral E1A also bind to cyclin A and cdk 2 (Green, 1989).

HPV16 E6 protein contains four presumed zinc binding motifs in its 158 amino acids. In common with adenovirus E1B and SV40 virus large T it binds to p53 - a protein which induces cell cycle arrest or apoptosis in response to DNA damage or inappropriate DNA synthesis. Transient replication assays suggest that p53 also suppresses HPV amplificational DNA replication (Lepik et al. 1998). Clearly p53 function needs to be overcome to allow productive HPV replication. E6 from high risk viruses achieves this by causing p53 to be degraded rapidly (Scheffner et al. 1990). E6 from low-risk PVs binds much less strongly to p53 and does not cause it to be degraded in *in vitro* systems.

E6 and E7 appear to be able to induce the synthesis of cellular DNA and the expression of cellular genes necessary for viral DNA replication. Until recently it has not been possible to study the function of E6 and E7 by genetic analysis in HPV culture systems because the existence of the cultures depended on the immortalising ability of these genes. Recently the problem has been avoided by using retrovirus-
mediated gene transfer into primary human keratinocytes which are then kept in an organotypic culture system (Chow and Broker 1997). There is a lot of host chromosomal DNA synthesis in the differentiated layers of condylomata and it has been shown using retrovirus-mediated transduction of HPV18E7 that E7 alone can induce this. It seems that E7 reactivates the host DNA replication machinery in differentiated cells and E6 prevents this from triggering apoptosis.

CIN and cervical cancer

For nearly 30 years pathologists have analysed cervical tissue as being on a continuum from normal epithelium through cervical intraepithelial neoplasia (CIN) to invasive cervical carcinoma (Richart 1968) although the assumption that there is a simple progression through these stages has been questioned (Kiviat, Critchlow, and Kurman 1992). It is now widely accepted that all CIN is a manifestation of HPV infection (Walboomers et al. 1994). Individuals with CIN1 (the least abnormal classification) rarely develop serious cervical pathology even without intervention and it probably represents transient HPV infection.

Screening by cytology

It is believed that population-wide screening programmes of cervical cytology have reduced the incidence of cervical carcinoma (Hakama, Miller, and Day 1986). Cervical carcinoma is particularly amenable to screening because the early stages all take place in the most superficial part of the cervix. This has two implications which together have allowed the population screening programme to be effective. Firstly, abnormal cells can simply be scraped off the cervix in a cervical "smear test" and later identified under the microscope. And secondly, because CIN remains entirely superficial for years it can be completely destroyed or excised by a simple procedure.

The cytologist is dependent on the quality of the specimen taken and this together with the subjective nature of the task result in the fact that cervical screening has less than 90% sensitivity (Koss 1989). This problem is largely overcome by regularly repeated
testing in the knowledge that the cancer usually takes 15 or more years to develop. As methods for detection of HPV become more reliable, it is beginning to be accepted that the presence or absence of oncogenic HPV in a cervical smear is a useful prognostic indicator (Cuzick et al. 1995).

**Immune response to viruses**

Every virus is uniquely adapted to its host so there can be no meaningful discussion of the immune response to a "typical" virus infection. Most of our knowledge comes from a relatively small range of virus infections which have been chosen either because of their convenience as a model to study in animals or because of their clinical importance as human pathogens. What all viruses have in common is that they are intracellular pathogens so although protection can be achieved by antibody alone, control or eradication of infection requires cellular effectors.

**Innate immunity to viruses**

The boundary between innate and adaptive immunity has become blurred now that it is known that antigen specific effectors often produce cytokines which activate local "innate" immunity.

**Interferons**

Interferon-α and interferon-β are produced by cells in response to infection with most, if not all, viruses. Double stranded RNA may be the common inducer. They act on virally infected cells via a surface receptor to promote the degradation of viral RNA or to inhibit its translation. Interferons α and β also upregulate MHC class I and elements of the class I processing pathway including TAP transporter proteins (Janeway and Travers 1996).
Natural killer cells

NK cells express receptors which recognise self MHC class 1 molecules and kill only cells which fail to express these. Many viruses act to decrease MHC class 1 expression on the cells they infect, this allows them to evade presentation to the adaptive immune system. As a result NK cells can specifically kill virally infected cells. NK cells are strongly activated by interferons α and β (Moretta, 1996).

Adaptive immunity to viruses

Antibodies

Viral infections may be prevented by virus-specific antibody. This is clearly demonstrated by the fact that hepatitis A can be prevented in travellers to endemic areas simply by the injection of pooled gamma globulin from healthy donors. Infection is only established if insufficient antibody is present.

Virus-specific lymphocytes

CD4 T cells clearly have an important role in immune responses to viruses but in the absence of a known mechanism by which they could directly eradicate or control infection they have been assigned a supporting role either as "helper" cells, a function they clearly have both in supporting B cell production of antibody (Parker 1993) and in supporting proliferation and action of CTL (Abbas, Murphy, and Sher 1996), or as "inflammatory" cells - able to activate macrophages and initiate a delayed type hypersensitivity response. CD4 T cells are also constrained by their requirement to encounter antigen on class II MHC which has only a limited tissue distribution. In recent years it has been realised that the response of CD4 T cells on first encountering antigen can determine the nature of an immune response and in particular whether a pathogen is controlled or not. The key is the pattern of cytokine production of the responding cells. Although the initially reported polarisation of
murine CD4 T cells into Th1 (producing IFNγ, IL2 and TNFβ) and Th2 (producing IL4, IL5, IL6 and IL13) subsets is not closely replicated in humans the principle that naive CD4 T cells can develop to produce different patterns of cytokines is maintained (Abbas, Murphy, and Sher 1996). The dominant pattern of cytokine production by these cells seems to be associated with, and presumably to cause, a particular immunological outcome. IFNγ and TNFβ are predominant in the control and clearance of most viral infections but host resistance also involves IFNα, IFNβ, natural killer cells and neutralising antibody. Furthermore, NK and CTL activation is unimpaired in mice lacking IFNγ, IFNγ receptor or IL2 (Graham et al. 1993, Huang et al. 1993, Kundig et al. 1993). It seems likely that viral immunity is not simply mediated by a polarised Th1-type response (Biron 1994).

For many years it has been believed that the chief way in which established viral infection is eradicated is by cytolysis of infected cells carried out by CD8 lymphocytes. This belief is supported by experimental evidence showing that these cells are the chief effectors in a long list of viral infections (Koszinowski, Reddehase, and Jonjic 1991). Although lysis of infected cells is important it is also now clear that anti-viral cytokines, produced by virus-primed lymphocytes, are also needed and in some cases may be sufficient for terminating infection. Clearance of hepatitis B virus from the liver is known to occur quite rapidly and is mediated by CD8+ T lymphocytes (Chisari and Ferrari 1995). Since virtually all hepatocytes are infected, clearance by cytolysis would lead to massive hepatic necrosis. Recent evidence has shown that viral clearance can be achieved in a hepatitis B transgenic mouse model by IFNγ and TNF α, local production of which is established by HBV-specific CTL which have been adoptively transferred (Guidotti et al. 1996b). It seems that these cytokines act against hepatitis B both by destabilising viral mRNA and by eliminating cytoplasmic viral nucleocapsids. Using the same model HBV could also be inactivated in the liver by IFNγ and TNF α produced locally by LCMV- infected macrophages (Guidotti et al. 1996a).
It seems there is no good reason why CD4 T cells alone cannot eradicate some viral infections. There is not always a requirement for cytolysis and anyway CD4 T cells often have cytolytic activity (Altmann et al. 1992). The limited tissue distribution of MHC class II can be overcome by IFNγ-induced MHC class II expression on infected cells. In several animal models if CD8 effectors are depleted their function can be replaced by CD4 T cells (Koszinowski, Reddehase, and Jonjic 1991). It is very likely that the nature of the CD4 T cell response to HPV16 determines the outcome of infection and it is possible that CD4 T cells are the main effectors.

**Immune response to papillomavirus infection**

*General considerations*

Any virus which successfully reproduces itself by establishing an infection and replicating within a host must be able to avoid the host's defences. This implies that as well as the standard virological requirements for cell entry, replication, assembly and release, the virus must be able to co-exist with the immune system of the host. Viruses employ a wide variety of methods to do this and understanding these methods sheds light on the workings of the immune system itself (Spriggs 1996). In general a virus might avoid triggering a systemic response at all, or it might evade effector mechanisms.

The strict epithelial tropism of papillomaviruses and their very restricted gene expression below the outer epithelial layers suggest that their main strategy is to remain invisible to the immune system. Keratinocytes are poor antigen presenting cells and presentation of HPV antigens on such cells may lead to tolerance because of inadequate co-stimulation (Bal et al. 1990). It remains possible that HPV gene products are also capable of interfering with immune responses. It has been reported that HPV16 E5 when transfected into keratinocytes causes post-transcriptional loss of TAP-1 and MHC class 1 heavy chain suggesting a mechanism to prevent
presentation of viral peptides on class 1 (McCance et al. 1995). This has not subsequently been confirmed.

**Natural history of papillomavirus infection**

Papillomaviruses are specialised to a particular type of epithelium in a particular species and DNA sequence comparisons suggest that they have evolved together with their host, its epithelium and its immune system (zur Hausen and de Villiers 1994). An implication of this is that data about papillomavirus immunity across species is of doubtful relevance and that in order to draw general conclusions about the natural history of a papillomavirus it is necessary to study it in its natural environment.

A distinction must be drawn between oncogenic HPV and other papillomaviruses as the methods used for studying the respective natural histories are quite different. Until recently our knowledge of the natural history of papillomavirus infection came solely from observation of large papillomas with the naked eye. In contrast oncogenic HPV tends to form only small flat papillomas, in women usually on the cervix, and can only be identified for certain by excision biopsy. Several large studies have recently examined the natural history of infection in cohorts of women by repeated assessment of cervical smears by PCR for specific viral types (Schiffman et al. 1996).

**Natural history of visible papillomas**

The classic natural history is that of a human skin wart which appears abruptly, sometimes at multiple sites, after an incubation period of at least three weeks. It persists with little visible change for a period of months or years and disappears abruptly, again sometimes from multiple sites (Oriel 1971, Rowson and Mahy 1967). A similar natural history has been reported for papillomas in cows (Jarrett 1985) and in rabbits (Kreider and Bartlett 1981) the chief differences are in length of persistence and chance of malignant transformation.
Natural history of oncogenic HPV

Large scale epidemiological studies in California, Oregon and elsewhere are currently investigating the natural history of oncogenic HPV infection as determined by the detection of the virus on cervical smears by PCR (Schiffman et al. 1996). It is clear that infection with these viruses is very common indeed and that in most women the virus becomes undetectable after a period of about one to two years. Cohorts have included women from an early age so as to include the ages of peak incidence which are known to be 18 - 24 and have found that between these ages at least 25% of women have detectable oncogenic HPV at some time. Persistence of the virus beyond 2 years is certainly a strong risk factor for the later detection of cytological abnormality but these studies cannot determine whether the virus is persistently detectable because CIN is already established or whether the persistence of viral infection leads to the development of CIN.

Immune response to papillomaviruses

Evidence of an immune response to an infecting agent in man is usually indirect. Epidemiological investigation of the natural history of infection, in particular clearance of the agent and protection from re-infection may be strong pointers. The detection of immune mediators primed to respond to the agent (usually specific antibody or primed lymphocytes) does not necessarily imply their efficacy. The best evidence can only come from altering the course of infection by intervention - vaccination or immunotherapy. The most encouraging evidence that there is an effective immune response to HPV comes from successful vaccination to prevent species-specific papillomavirus infection in cattle, rabbits and dogs.

Immune response to bovine papillomavirus

Two types of BPV are known to predispose cattle to cancer: BPV2 and BPV4. Vaccination with BPV2 L1 as a bacterial fusion protein could protect cattle from
challenge with BPV2 (Jarrett et al. 1991). Cattle could be protected from challenge with BPV4 by vaccination with the minor capsid protein L2 alone (Campo et al. 1993). In these experiments protection was accompanied by the presence of circulating antibody which neutralised live virus \textit{in vitro}. However protection by transfer of antibody has not been demonstrated.

Although vaccination with BPV4 E7 fusion protein did not protect animals from infection and tumour formation it did lead to early regression of tumours (Campo et al. 1993). It is not clear how this effect was mediated. Both antibody and cellular responses to BPV4 E7 were apparently stronger than in naturally infected cattle following papilloma regression (Chandrachud et al. 1994, McGarvie et al. 1995). Resolving papillomas in cattle have prominent mononuclear cell infiltrates but the nature of these cells has not yet been examined in this system.

These findings suggest that antibody to the capsid protein of BPV2 or BPV4 may alone be sufficient to protect cattle from infectious challenge with the whole virus. However although BPV2 and BPV4 are closely related there was no cross protection between viral types. The findings from vaccination with BPV4 E7 suggest that resolution of BPV4 papillomas may involve immune responses to E7 and that they are mediated by a poorly defined mechanism probably not involving antibody.

\textit{Immune response to cottontail rabbit papillomavirus}

CRPV has long been established as a model papillomavirus infection. Both spontaneous regression and progression to cancer are seen in some infected individuals (Kreider and Bartlett 1981) and, as proposed for HPV16, this has been shown to have an association with particular class II alleles (Han et al. 1992). Rabbits can be protected from infectious challenge with CRPV by immunisation with L1 or L2 as bacterial fusion proteins (Lin et al. 1992), with recombinant vaccinia expressing L1 (Lin et al. 1992), or with virus-like particles containing L1 alone (Jansen et al. 1995) or both L1 and L2 (Breitburd et al. 1995). Immunisation
with E1 or E2 as fusion proteins did not prevent infection but led to accelerated regression (Selvakumar et al. 1995).

More recently immunisation of rabbits with a polynucleotide vaccine expressing CRPV L1 was shown to protect from infectious challenge. This protection could be transferred to a naive rabbit by infusion of immune serum (Donnelly et al. 1996).

Immune response to canine oral papillomavirus

Canine oral papillomavirus causes mucosal papillomas in the mouths of beagles and has recently been used as a further species-specific model for HPV infection. The natural history of the experimental infection is of spontaneous regression after about 3 weeks (Suzich et al. 1995). As in the other animal models vaccination with wart extract could protect the animal from challenge with live virus (Bell et al. 1994). Interestingly if this extract was not formalin inactivated it caused squamous carcinoma at the site of infection in a small proportion of dogs. Vaccination was also successful with virus-like particles consisting only of L1 protein, in these experiments protection could be transferred to unvaccinated animals by infusion of serum protein alone (Suzich et al. 1995).

In this model it appears that the L1 protein needs to be in the form of VLP as denatured antigen did not protect. There is no published information on the immunological events accompanying regression of COPV papillomas.

Immune response to human papillomavirus

Immune response to human papillomavirus in animals

The strict tissue and species tropism of HPV means that it does not naturally infect other species. Nevertheless given the difficulties in studying HPV immunology it is reassuring that HPV proteins seem to be immunogenic in laboratory mice. Epitopes restricted by murine class I (Beverley et al. 1994) and class II (Tindle et al. 1991) have been defined (see chapter 5). It has been shown in mice that immunisation with
HPV16 E7 can lead to rejection of a tumour expressing this antigen (Chen et al. 1991) and in rats the same was achieved with a combination of E6 and E7 (Meneguzzi et al. 1991). Since all HPV-associated cervical tumours continuously express these antigens hopes have been raised for successful immunotherapy. Of course their immunogenicity in rodents does not necessarily imply that these proteins are similarly immunogenic in humans.

*The human immune response to human papillomavirus*

The definitive demonstration that an effective immune response to HPV is possible would be successful vaccination. Until then more indirect evidence must be examined. The field of HPV immunology is made difficult by the fact that the virus cannot easily be produced *in vitro* so studies have to be carried out on individual viral proteins synthesised in the laboratory. Serological studies have improved enormously now that virus-like particles can be used as these have the correct conformation to allow antibody recognition.

*Clinical observations*

It is frequently observed that multiple warts regress simultaneously suggesting a systemic immune response and histological study of regressing warts shows an influx of macrophages and lymphocytes (Stanley, Coleman, and Chambers 1994). The lymphocytes are chiefly CD4 T cells and the appearance is of a delayed-type hypersensitivity response.

While individuals with profound antibody deficiencies do not have an increase in HPV disease, it is clear that defects in cellular immunity, whether through immunosuppression or HIV infection, do lead to a greater prevalence of warts (Frazer and Tindle 1992). In addition renal transplant patients, who have a prolonged period of immunosuppression, have a greater frequency of HPV associated tumours including both squamous carcinoma of the skin and cervical carcinoma (Sheil 1992). However there is a much greater increase in the incidence of other virally induced
tumours including Hodgkin's lymphoma. This suggests that immune surveillance of latent EBV infection is of more importance. It may reflect the fact that EBV latency is both common and life-long whereas HPV infections may be more transient.

**Skin testing**

In a small study 6 out of 7 women with CIN and detectable HPV16 or 18 gave a classic "delayed type hypersensitivity" response to intradermal injection of HPV16 L1 protein compared with 0 of 10 control individuals. This suggests the presence of circulating primed CD4 T cells capable of responding to HPV16 virions (Hopfl et al. 1991).

**HLA association (host variation)**

In 1991 Wank and Thomssen compared the serologically defined HLA types of 66 cervical carcinoma patients with those of a panel of 2019 uncharacterised population controls and claimed that women expressing HLA Dw3 are more likely to develop cervical carcinoma (Wank and Thomssen 1991). This was refined by PCR-based HLA typing to show an increased risk for DQB1*0301 and 0303 (Wank, Schendel, and Thomssen 1992). Subsequently numerous other studies have been conducted looking for such associations. They have produced differing and sometimes contradictory results. Some have confirmed an association with DQB1*03 (Gregoire et al. 1994, Helland et al. 1994, Odunsi et al. 1995), others failed to find this and some reported other associations (Apple et al. 1994, Glew, Duggan-Keen, and Ghosh 1993).

That these studies vary in their conclusions is perhaps not surprising. The numbers of subjects included have generally been small so they have insufficient power to detect unequivocally what may be only weak associations. Studies have often used quite different definitions of patients and controls who come from a variety of racial groups with differing background HLA prevalences. The transition from HPV
infection to CIN could have a different HLA association from the transition from CIN to carcinoma.

Some of these studies consider each inferred haplotype separately rather than as one of a pair expressed by an individual and nearly all consider each allele separately rather than as a group co-expressed by an individual. This approach could miss specific DQ associations. Only one part of a functional DR heterodimer is polymorphic (DRB) so DR associations can be established by this method. However both DQA and DQB are polymorphic and the alleles are all co-expressed (Janeway and Travers 1996). Each individual can have four different DQ molecules and if only single haplotypes are analysed half of these DQ combinations will be missed. This is not just a theoretical issue. The principle has been clearly demonstrated in coeliac disease (Sollid et al. 1989). A single haplotype HLA-DR3DQw2 has a strong association with the disease. Sollid et al demonstrated that nearly all individuals with coeliac disease who do not have this haplotype nevertheless express the same combination of DQA1 and DQB1 on different haplotypes.

Class II associations with HPV disease could result from linkage disequilibrium with a susceptibility factor (as yet unidentified), in which case the association will be with specific haplotypes, or they could be mediated by the class II molecules themselves. The latter possibility would suggest that CD4 T cells have a central role in the outcome of infection. Even the strongest associations confer only a modest relative risk and other variables will have an influence. As well as environmental factors it is likely that non MHC genetic factors are involved as has been shown for type 1 diabetes (Davies et al. 1994). Even if an HLA allele does itself confer risk or protection it is not obvious how it would do so. It would seem to imply that the outcome of the immune response to HPV depends critically on the interaction of the chosen allele with one or a small number of peptide epitopes. If this is the case protection could be conferred if presentation of this epitope by the class II molecule encoded by the allele resulted in control of the virus. Susceptibility perhaps would
require alteration in the T cell repertoire by the negative selection of particular T cell specificities in the thymus. There is no evidence supporting this.

One paper claims to have shown that women with HPV16 associated cervical carcinoma are more likely to have a mutation in a CTL epitope presented on HLA B7 (Ellis et al. 1995). The only evidence that this peptide sequence is indeed such an epitope comes from in vitro binding studies. There have been no reports of CTL generated by this peptide and there is no evidence that such a response is important for control of the virus. Indeed, as discussed later, the demonstration of CTL naturally primed by HPV16 has proved very difficult. The wider question of HPV16 variants and cancer risk has also been addressed.

*Mutant virus (virus variation)*

It has been claimed that among women with HPV16 infection individuals with cervical carcinoma are more likely to carry virus with certain mutations than individuals with milder disease (Ellis et al. 1995, Londesborough et al. 1996). A recent international analysis of HPV16 sequence variation supports earlier suggestions that most variants tend to cluster in specific geographical locations (Yamada et al. 1997). Too little is known about these variants and the specificity of the immune response to HPV16 to say whether these findings have important implications for vaccination strategies.

*Serology*

Until recently most attempts to study the prevalence and specificity of HPV antibodies in the sera of infected individuals were severely hampered by difficulty in obtaining satisfactory antigen. At first it was not known that different types of HPV existed, pooled virions from skin warts were used and gave inconclusive results. Later, type specific antigens were used. HPV1 virions are relatively easy to obtain from clinical material and using these it became clear that antibody to
conformationally correct antigen on native virions correlated with disease status far better than antibody to disrupted virions (Steele and Gallimore 1990).

At this time conformationally correct genital HPV material was not available. Studies were conducted using fusion proteins or synthetic peptides and although attempts were made to distinguish relevant antibody the results remained inconclusive (Galloway 1992). Now that self-assembled VLP have been manufactured, larger scale serology studies are being repeated for oncogenic HPV. About 60% of women with currently detectable HPV16 DNA have antibody (Kimbauer et al. 1994) and most, but not all, women who acquired HPV16 during a cohort study developed antibody after a delay of perhaps four or five months (Wikstrom et al. 1995). However only about half of women with HPV16 positive invasive carcinoma have detectable antibody (Nonnenmacher et al. 1995). It seems unlikely that serology will become a clinical tool to rival cervical cytology in detecting women at risk of developing cervical cancer.

The antibodies in these studies are to the capsid proteins L1 and L2. These are the only viral products exposed on the surface of the infectious virions and antibodies to these proteins have been shown to protect from infection in the animal models described above. Antibody to the early proteins E6 or E7 can be detected in up to a third of women with cervical carcinoma (Nindl et al. 1994). There is no indication that they have any prognostic significance and they are too uncommon to be diagnostically useful.

Cellular immunity

CD4

The likely, and often underestimated, importance of CD4 T cells in controlling viral infections has been described above. This is particularly true for HPV infection where the available evidence suggests that CD4 T cells are more important as effectors than class I restricted CTL. The observations which suggest this are the
presence in resolving papillomas of an infiltrate consisting mainly of macrophages and CD4 T cells, the numerous reports suggesting a class II association with HPV disease both in humans and in rabbits and the increased prevalence of HPV disease in patients with HIV infection where immune deficiency seems to centre on CD4 T cells. In spite of this it has consistently proved difficult even to demonstrate the existence of such primed cells in individuals infected with HPV16 and their cytokine-producing phenotype is unknown. As this is the main focus of this thesis it is discussed in detail in chapter 3 (human responses), chapter 4 (murine responses) and in the final discussion.

CD8 (CTL)

Although there is no clear evidence that control or eradication of HPV requires cytolysis by class I restricted T cells, CTL are known to be important effectors in other viral infections and against some tumours (Boon et al. 1994, Koszinowski, Reddehase, and Jonjic 1991). It has proved very difficult to demonstrate CTL primed to HPV16 in the peripheral blood or in lymph nodes draining cervical cancers using methods which were successful for influenza (McIndoe 1993). It has been shown however that it is possible to prime such cells in vitro. Using E7 peptides chosen in an HLA A2 binding assay CTL were generated from PBMC of healthy donors that were capable of lysing cells from an E7 expressing cervical cancer line (Ressing et al. 1995). CTL specific for HPV18 were obtained from one subject of a phase 1/11 trial of vaccination against HPV16 and HPV18. The vaccine was recombinant vaccinia expressing E6 and E7 from both HPV16 and 18 (Borysiewicz et al. 1996).

In summary, although the nature, prevalence and specificity of antibody responses to HPV16 are beginning to become clear, very little is known about the cellular component of the natural immune response.
Chapter 2

Materials
Table 2.1  Plastics

<table>
<thead>
<tr>
<th>Description</th>
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<td>7ml Bijou containers</td>
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Media

Cell culture media:

RPMI: RPMI 1640 supplemented with 2g/litre sodium bicarbonate and 2mM glutamine (ICRF media production).

IMDM: Iscove's modified Dulbecco's medium (Gibco) supplemented as RPMI (ICRF media production).

E4 (DMEM) Dulbecco's modification of Eagle's medium (ICRF media production).

HL1: serum free medium (New Brunswick Scientific, Hatfield, UK)

Sera:

Fetal calf serum (FCS, Gibco) was routinely heat inactivated at 56° for one hour before freezing in aliquots. A specific batch of FCS was used for all murine
lymphoproliferation assays because it was found to give consistently low background proliferation.

Pooled AB+ human serum (National blood transfusion service) was heat inactivated in the same way and frozen in aliquots.

_Bacterial growth media_

LB: Bacto-Tryptone 100g, Yeast extract 50g, NaCl 100g in 10 litres H₂O (ICRF media production).

Buffers and solutions

Water

Water was purified by a Millipore reverse osmosis system and then polished to give water at < 10MΩ.cm³

PBSA: Phosphate buffered saline:

8g/l (137mM) NaCl

0.25g/l (3mM) KCl

1.43g/l (10mM) Na₂HPO₄

0.25g/l (2mM) KH₂PO₄

TE:

10mM Tris-HCl, pH 8.0

1mM EDTA, pH 8.0
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<tr>
<td>Isopropanol</td>
<td>BDH</td>
</tr>
<tr>
<td>KCl</td>
<td>BDH</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>BDH</td>
</tr>
<tr>
<td>KLH</td>
<td>Sigma</td>
</tr>
<tr>
<td>Methanol</td>
<td>BDH</td>
</tr>
<tr>
<td>Milk powder</td>
<td>Tesco</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>Sigma</td>
</tr>
<tr>
<td>Mycophenolic acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>NaCl</td>
<td>BDH</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>BDH</td>
</tr>
<tr>
<td>NaN$_3$</td>
<td>Sigma</td>
</tr>
<tr>
<td>Ovalbumin Grade VII</td>
<td>Sigma</td>
</tr>
<tr>
<td>PHA</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
PPD
Protogel (30% w/v acrylamide, 0.8% w/v bisacrylamide)  National Diagnostics
SDS  BDH
Thymidine (tritiated)  Amersham
Trizma base  Sigma
Trypan blue (0.4% solution)  Sigma

Table 2.3  Restriction enzymes and modifying enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI (20,000U/ml)</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>DNA ligase (high concentration)</td>
<td>Gibco</td>
</tr>
<tr>
<td>EcoRI (25,000U/ml)</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>HindIII (18,000U/ml)</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>Klenow fragment of DNA Polymerase I</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>NotI (20,000U/ml)</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>PstI (15,000U/ml)</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>SmaI (9000U/ml)</td>
<td>Pharmacia</td>
</tr>
</tbody>
</table>
Table 2.4  Stock solutions of cell culture reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Preparation and storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdU</td>
<td>2.5 mg/ml in PBS</td>
</tr>
<tr>
<td></td>
<td>Filtered through 0.2μm and stored in aliquots at 4°C protected from light.</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>5 x 10⁻³ M in IMDM</td>
</tr>
<tr>
<td></td>
<td>Filtered through 0.2μm and stored in aliquots at -20°C</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>1 mg/ml in PBS</td>
</tr>
<tr>
<td></td>
<td>Filtered through 0.2μm and stored in aliquots at 4°C protected from light for maximum 2 weeks.</td>
</tr>
</tbody>
</table>

Protein gel electrophoresis (SDS-PAGE)

The following ingredients were used for the gels and buffers:
Acrylamide: 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide (National diagnostics).
Resolving gel buffer: 1.5M Tris pH 8.8, 0.1% SDS (final concentration in gel).
Stacking gel buffer: 0.5M Tris pH 6.8, 0.1% SDS (final concentration in gel).
10% Ammonium persulphate 1/200 final volume of gel.
Temed (N,N,N', N'-Tetra methylethlenediamine) (Gibco) 1/2000 final volume of gel. 
H₂O to make up volume.

Sample loading buffer (for 10ml of x 10): 6.85ml 1.0M Tris pH 6.8, 1g SDS, 3ml glycerol, 1ml β mercaptoethanol, bromophenol blue.

Running buffer (x 10): 144g Glycine, 30g Tris base, 10g SDS, dissolved in 1 litre H₂O.

*Peptides*

Peptides were purchased from Dr M Mumms (Peptide and protein research consultants, Exeter). They were synthesised on manual solid-phase machines, purified chromatographically and analysed with slow gradient HPLC and mass spectroscopy. Purity was greater than 80%. Sequences and their relation to the sequence of HPV16 L1 and E7 are given in tables 2.1 and 2.2. Peptides were stored in powder form at -20°C or dissolved in DMSO at 20mM.
Figure 2.1

HPV16 E7: the full sequence and the set of overlapping peptides
MQVTFIYILVITCYENDVNVDHF
QMSLWLPSSEATVYLPPVPVSKVST
DEYVARTNIYYHAGTSRLLAVGHPY
FPIKKPNNNKILVPKSGLQYRFR
IHLPDPNKFGFPTSFYNPDTQRL
WACVGVGVGRQPGLGVGISGHPLL
KLDSTDENASAYAANAGVDNRECISM
DYKQTQLCLIGCKPPIGEHWKGSP
CTNVAVNPGDCPPLELINTVIPGD
MVHTGFGAMDFTTLQANKSEVPLDI
CTSICKPyPDYIKMVSEPYGDSLFFY
LRREQMFRHLFNRAGTVGENVPDD
LYIKGSSTANLASTNYSYFTPSDSM
UTSDAQIFNKPYWLQRAQGHNNNGIC
WGNQLFVTVDTDTRSTNMCLAAIS
TSETTYKNTNFKEYLRGEEYDLQF
IFQLCKITLTADVMTYIHSMNSTIL
EDWNFGLQPQPGTLEDYRFVTQAI
IACQKHPPAPKEDDPLKYYTFWEV
NLKEKFSADLDQFPLGRKFLLOAGL
KAKPKFTLGKAKATPTTSSTTTAK
RKKRKL

Figure 2.2: HPV16 L1: The full sequence. The parts in bold type show the region covered by the overlapping peptides
Figure 2.3

Amino acid sequence of the HPV16 L1 peptides
Table 2.5 List of antibodies used and their source

All of these antibodies were gifts.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Specificity</th>
<th>Originator</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN130</td>
<td>Mouse anti human CD45RA</td>
<td>G Janossy, Royal Free</td>
</tr>
<tr>
<td>UCHL1</td>
<td>Mouse anti human CD45RO</td>
<td>P. Beverley</td>
</tr>
<tr>
<td>QS4120</td>
<td>Mouse anti human T cell subset CD4</td>
<td>Q. Sattentau, Marseilles</td>
</tr>
<tr>
<td>OKM1</td>
<td>Mouse anti integrin α subunit CD11b</td>
<td>P. Kung</td>
</tr>
<tr>
<td>BU12</td>
<td>Mouse anti human B cells (CD19)</td>
<td>I MacLennan, Birmingham</td>
</tr>
<tr>
<td>UCHT4</td>
<td>Mouse anti human T cells subset CD8</td>
<td>P. Beverley</td>
</tr>
<tr>
<td>CAMVIR 4</td>
<td>Mouse anti HPV16 E7</td>
<td>M. Stanley, Cambridge</td>
</tr>
</tbody>
</table>
Chapter 3

Detection of memory CD4 lymphocytes specific for HPV16 in humans
Aim

The aim of this work was to detect and analyse class II restricted CD4 lymphocytes which had been primed by natural infection with HPV16. The evidence reviewed in the introduction suggests that HPV infection is controlled or eradicated by an immune response and that a major mechanism is a delayed-type hypersensitivity response orchestrated by CD4 T cells.

It was planned to find individuals who have been exposed to HPV 16 infection, to find a way of detecting specific memory/effector CD4 T cells and then, having isolated such cells, to analyse their epitope specificity, MHC restriction and cytokine production. It is not possible to demonstrate that such cells are capable of eradicating or attenuating the infection but their presence in individuals who have had resolution of infection or of CIN would at least be suggestive of this.

Detection of human CD4 T cells primed by viral infections

Although it is commonly assumed that there are three phenotypic forms of CD4 T cells which are capable of recognising a particular antigen: naive cells, effector cells and memory cells, in practice these phenotypes are not clearly distinguishable. Although there are surface markers which seem capable of distinguishing naive cells (which have not encountered antigen) from memory/effector cells (which have encountered antigen) (Merkenschlager et al. 1988b) "memory" markers may later revert to the "naive" pattern blurring this distinction. In addition there is at present no reliable way of distinguishing memory cells from effector cells either in humans or in other animals. In the future cytoplasmic staining for cytokines may provide a way of distinguishing CD4 T cells which are producing cytokines (effector cells) from cells which merely have the ability to start producing cytokines quickly when activated (memory cells) (Carter and Swain 1997). However it remains possible that
memory cells and effector cells are indistinguishable functionally (Zinkernagel et al. 1996).

It is characteristic of a recall immune response that it is both faster and stronger than a primary response. For CD4 T cells this implies that there is a rapid clonal expansion of memory cells to produce many effectors. In principle such a response can be detected by assessing cellular proliferation in the presence of specific antigen. Although this is a very indirect measure which will not always accurately reflect specific T cell activation, it has the advantage of simplicity. Alternatively it is possible to look at the effector function of the responding cells (for instance help for antibody production (Gray and Matzinger 1991)) or at other measures of T cell activation in the presence of specific antigen (McHeyzer-Williams and Davis 1995) but these are hard to address in humans. Now that the heterogeneity of human CD4 T cell responses has been established it is important to try to define the cytokines produced by responding T cells (Abbas, Murphy, and Sher 1996). The most effective way of doing this is to clone the cells before measuring cytokine production but this may tend to over represent those T cells which are most easily cloned (Gammon et al. 1990). The alternative approach of measuring the cytokine production of cells in bulk culture will give only a "consensus" result which could miss a small but important sub population. Some of these problems may be overcome by the newer approach of intracellular staining for cytokines (Carter and Swain 1997). For instance by stimulating PBMC with a viral antigen and then shortly afterwards co-staining for an activation marker (such as IL2 receptor) and intracellular cytokine production. At present this technique is probably too insensitive to detect low frequency responses.

Early studies of cellular immunity assessed specific activation by examining cells and looking at the frequency of blast transformation. A simpler approach to detect such a response, and the method used in this work, is to quantify cellular proliferation by culturing cells with specific antigen then pulsing with $[^{3}\text{H}]T\text{dR}$. 

60
[\textsuperscript{3}H]TdR is rapidly transported into cells where it is phosphorylated and becomes thymidine triphosphate, an immediate precursor of DNA. The triphosphate is not readily transported across cell membranes. Some of the theoretical ways in which [\textsuperscript{3}H]TdR incorporation into cells may not reflect cellular proliferation have been discussed by Ashman (Ashman 1984). These include the possibility of differences between cell populations in the rate of [\textsuperscript{3}H]TdR uptake, differences in distribution of [\textsuperscript{3}H]TdR between intracellular compartments or different rates of phosphorylation within cells. Incorporation of [\textsuperscript{3}H]TdR into DNA could reflect DNA repair rather than replication. Although there is evidence using mitogen-stimulated murine and human lymphocytes that proliferation reflects activation quite closely (Buckley and Wedner 1978), for antigen-specific cellular activation the delay of days before proliferation leaves more time for the factors above to cause problems and sometimes activation can lead to early effects like cytokine production without causing appreciable proliferation at all (Evavold and Allen 1991). Other potential problems include the presence of marked although variable thymidine phosphorylase activity in whole blood, in some serum and extracellularly in association with some blood cells (Bodycote and Wolff 1986). If [\textsuperscript{3}H]TdR is phosphorylated extracellularly it is not available for uptake into cells. Finally there is great difficulty, particularly when using this simple method, in distinguishing between primary and secondary responses. This will be discussed later.

**Problems specific to HPV16**

*Source of antigen*

There remains no way of producing HPV16 virions *in vitro* in sufficient quantity for immunological studies. It is possible to extract adequate material from plantar warts to investigate immune responses to HPV1 (Steele, Stankovic, and Gallimore 1993) but the papillomas caused by HPV16 are much smaller. Protein or peptide corresponding to the known sequence has to be produced *in vitro*. Although protein to stimulate CD4 T cells does not need to be conformationally correct it does need to
be very pure. HPV16 proteins have been produced in bacteria as fusion proteins (Banks et al. 1987, Cubie et al. 1989), in yeast (Carter et al. 1991, Tommasino et al. 1990), and as baculovirus constructs in insect cells (Stacey et al. 1992). They have also been produced by recombinant vaccinia virus (Browne et al. 1988, Zhou et al. 1991b) and as overlapping series of peptides (Comerford et al. 1991, Tindle et al. 1991).

Since a considerable investment of time and effort is needed to produce just one HPV16 protein by any of these methods it is not feasible to attempt to use a combination of the products of all 7 putative ORFs so it is necessary to decide which to study. The problem is particularly severe when using peptides as the cost of producing sufficient peptides to cover only a small region of protein is high. The immune response to pathogens usually seems to be focused on only a small number of immunodominant epitopes (Sercarz et al. 1993). This is probably the case for HPV16 so the correct choice of ORF could be essential.

Source of cells

HPV16 infection is always very localised and it is likely that the initial expansion of primed T cells will be in the local lymph nodes. However since it is not possible in practice to obtain such nodes other than from patients at the time of operation for cervical carcinoma it is usually necessary to compromise by looking in the peripheral blood.

If indeed there is a systemic immune response to HPV16 and primed lymphocytes do recirculate in the peripheral blood it is still necessary to decide which people to test and when to test them. In women with CIN or cervical carcinoma and persistent HPV16 it is possible that viral persistence demonstrates the failure of the immune response. Conversely individuals who have only a mild and transient infection may have had insufficient exposure to the viral antigens for primed lymphocytes to be detectable in the blood.
Materials and methods

Human subjects

*Laboratory donors*

These fifteen donors were uncharacterised for HPV16 infection, all were aged between 21 and 40, 5 were male and 10 were female. These donors are identified by a letter of the alphabet with the prefix "L". 60ml peripheral blood was taken from each for every experiment.

*Patients*

Patients were recruited from two sources. The colposcopy clinic of the department of sexually transmitted disease at University College London (Mortimer Market Centre) and the gynaecology clinic of the Whittington hospital. The aim was to take blood from patients who had tested positive for HPV16 on the cervix by type-specific PCR.

*Mortimer Market Centre*

The Mortimer Market Centre is the sexually transmitted disease clinic of University College Hospital, London. If a routine cervical smear test shows abnormal cytology which requires further investigation the patient is referred to the colposcopy clinic. A protocol was set up to recruit patients from this clinic and was approved by the local ethics committee. When a patient attended for colposcopy she was informed of the study and asked if she was willing to take part. Those who agreed had a further cervical smear test performed immediately before colposcopy. The spatula was swirled in 5ml PBS which was immediately frozen. At a later date DNA was extracted from this buffer and tested for HPV16 by type-specific PCR (Terry *et al.* 1993). This testing was carried out in the laboratory of Linda Ho and George Terry at University College London. Patients who tested positive for HPV16 were recalled and 30 - 60 ml blood was taken for use in a proliferation assay. A total of 144 patients were screened, blood was taken from 14 of the 31 found to have detectable
HPV16. These subjects are identified by a letter of the alphabet with the prefix "M".

Their histological diagnoses following colposcopy are shown in table 3.3

Whittington Hospital

Under a previously established protocol patients referred to the colposcopy clinic run by the department of women's health at the Whittington Hospital, London were screened for oncogenic papillomaviruses on the cervix by the method described above. On their subsequent attendance some patients who had been previously found positive for HPV16 were informed of this study. 30 - 60 ml blood was taken from those who agreed to take part. 12 patients were recruited in this way and are identified by a letter of the alphabet with the prefix "W". Their histological diagnoses following colposcopy are shown in table 3.2

Preparation of peripheral blood mononuclear cells

Blood was heparinised at venesection by the addition of a trace of heparin (1000 U/ml) to the syringe. Heparinised venous blood was processed within six hours of bleeding. The blood was diluted 1:1 with PBSA and layered on to ficoll hypaque. This was spun at 1100 x g for 20 minutes without braking. The layer of cells at the interface of aqueous and Ficoll layers was removed and washed twice in PBSA before being resuspended in the appropriate medium.

Cell counting

An aliquot of 20μl of cell suspension was diluted 1:1 with trypan blue solution (0.2% w/v in PBS with 3mM NaN₃) The cells were then viewed and counted on an improved Neubauer counting chamber with a light microscope. Only cells which excluded trypan blue were counted.
Adherence

In some experiments PBMC were separated into populations according to their ability to adhere to plastic. Whole PBMC were incubated for 1 hour in a total volume of less than 10ml in a plastic culture flask in a CO\textsubscript{2} incubator. Non-adherent cells were then tipped off, washed in medium and resuspended. Adherent cells were removed using a plastic cell scraper.

Subfractionation of PBMC

PBMC were enriched for certain sub-populations by depletion using specific antibodies and magnetic beads with immobilised anti murine IgG (Dynabeads, Dynal) according to the manufacturer’s instructions. All manipulations were carried out on ice or at 4°C. Non-adherent PBMC were cultured with optimal dilution (previously determined) of the appropriate murine anti human antibody (see table 2.5) on ice for one hour then washed three times with PBS. Dynabeads were washed with PBS to remove azide then added to the labelled cells (at a ratio of 4 per cell) incubated on ice for 30 minutes with continual mixing then removed by using a magnet to immobilise the beads and pipetting out the supernatant. Two cycles of depletion with Dynabeads were performed.

Treatment of cells with mitomycin C

10^7 cells were incubated with 100\mu g mitomycin C in a volume of 1ml for 1 hour at 37°C. The cells were then washed x3 with IMDM.

Proliferation assays

Whole or subfractionated PBMCs (1 - 2.5 \times 10^5 per well) were cultured with antigen in 96 well round-bottom plates in a total volume of 200\mu l per well of IMDM with 5% human serum. When subfractionated cells were used, fresh autologous adherent cells were added as APCs (10% of responder cell number). Controls with no antigen
and with PHA at 1 μg/ml were included in each assay. The plates were incubated for 5 -6 days at 37° in a humidified atmosphere with 5% CO₂. They were then pulsed for 6 -16 hours with 10μl PBSA per well containing 1μCi [³H]TdR before being harvested onto glass fibre filters using a cell harvester (Skatron). Incorporation of [³H]TdR was assessed by liquid scintillation using the LKB betaplate system. All points were performed in triplicate and the mean of triplicate results is shown for all results unless described otherwise. Experiments with excessive variation between triplicates (standard deviation greater than 15% of the mean) were discarded.

Production of bacterial fusion proteins

pGEX system

Proteins are expressed fused to glutathione S transferase from Schistosoma japonicum with a site for cleavage by factor Xa between the protein and the fusion partner. GST fusion proteins were produced according to the manufacturers instructions (Pharmacia) with adaptations described below. Sonication was performed using a Branson sonicator. The constructs used in this work were all obtained from other laboratories and are detailed in table 3.1.

Cleavage of fusion partner from HPV16 protein using factor Xa

75 μg fusion protein was incubated with 1 μg of factor Xa in the presence of 1mM calcium chloride overnight at 4°C. Total reaction volume was 250 μl.

Protein quantitation

Protein quantitation was achieved using a BCA protein assay kit according to the manufacturer's protocol (Pierce).
Table 3.1  pGEX plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Insert</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEX2T</td>
<td>No insert</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pGEX6</td>
<td>HPV16E6</td>
<td>Gift from K Vousden</td>
</tr>
<tr>
<td>pGEX7</td>
<td>HPV16E7</td>
<td>Gift from K Vousden</td>
</tr>
<tr>
<td>pGEX4</td>
<td>HPV16E1^E4</td>
<td>Gift from J Doorbar</td>
</tr>
<tr>
<td>pGEXL1</td>
<td>HPV16L1</td>
<td>Gift from L Crawford</td>
</tr>
</tbody>
</table>
Protein gel electrophoresis (SDS-PAGE)

Proteins were separated by SDS-PAGE by the method of Laemmli (1970). 10% and 15% acrylamide gels were used. The composition of gels and buffers is described in chapter 2.

Peptides

Peptides were obtained from Dr M Mumms (Peptide and protein research consultants, Exeter). They were synthesised on manual solid-phase machines, purified chromatographically and analysed with slow gradient HPLC and mass spectroscopy. Purity was greater than 80%. Sequences and their relation to the sequence of HPV16 L1 and E7 are given in figures 2.1, 2.2 and 2.3. Peptides were stored in powder form at -20°C or dissolved in DMSO at 20mM.

Results

Mortimer Market Centre

31 (22%) of 144 screened patients had HPV16 detectable on the cervix. 14 of the HPV16 positive patients returned for blood tests. Colposcopy was performed immediately after screening and the histological diagnoses are given in table 3.3.
<table>
<thead>
<tr>
<th>Patient code</th>
<th>Age when tested</th>
<th>Histological diagnosis following colposcopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>WA</td>
<td>28</td>
<td>CIN 11</td>
</tr>
<tr>
<td>WB</td>
<td>31</td>
<td>CIN 111</td>
</tr>
<tr>
<td>WC</td>
<td>22</td>
<td>CIN 1</td>
</tr>
<tr>
<td>WD</td>
<td>24</td>
<td>CIN 11</td>
</tr>
<tr>
<td>WE</td>
<td>25</td>
<td>No abnormality.</td>
</tr>
<tr>
<td>WF</td>
<td>31</td>
<td>CIN 111</td>
</tr>
<tr>
<td>WG</td>
<td>52</td>
<td>CIN 1</td>
</tr>
<tr>
<td>WH</td>
<td>38</td>
<td>CIN 111</td>
</tr>
<tr>
<td>WI</td>
<td>28</td>
<td>Cervical papilloma.</td>
</tr>
<tr>
<td>WJ</td>
<td>24</td>
<td>CIN 1</td>
</tr>
<tr>
<td>WK</td>
<td>25</td>
<td>CIN 1</td>
</tr>
<tr>
<td>WL</td>
<td>23</td>
<td>Mild cytological dysplasia, no biopsy taken.</td>
</tr>
</tbody>
</table>
Bacterial fusion proteins: purity and yield

pGEX plasmids: GST fusions

The yield and purity of the fusion proteins varied considerably. None gave a single band on SDS PAGE (plate 3.1). Yields were improved by growing the bacteria at 30°C instead of 37°C and by altering the length of sonication, but always remained low. Whereas 1 litre of culture of pGEX2T could produce 18 mg of GST, 1 litre of pGEX with any of the HPV16 constructs produced at best between 1 and 2 mg of fusion protein - the majority of which was GST. Although the constructs used had a site for cleavage by factor Xa between GST and the fusion partner, attempts to cleave E6 or E7 from GST and to repurify resulted in negligible quantities of the HPV16 protein (results not shown).

Because of the difficulties in cleaving the proteins from their fusion partners it was decided to use the fusion proteins in proliferation assays with GST alone as a control antigen.
<table>
<thead>
<tr>
<th>Patient code</th>
<th>Age when tested</th>
<th>Histological diagnosis following colposcopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA</td>
<td>26</td>
<td>CIN 111</td>
</tr>
<tr>
<td>MB</td>
<td>24</td>
<td>CIN 1</td>
</tr>
<tr>
<td>MC</td>
<td>27</td>
<td>CIN 111</td>
</tr>
<tr>
<td>MD</td>
<td>25</td>
<td>CIN 11</td>
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<tr>
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<td>24</td>
<td>CIN 1</td>
</tr>
<tr>
<td>MG</td>
<td>30</td>
<td>CIN 11</td>
</tr>
<tr>
<td>MH</td>
<td>23</td>
<td>CIN 111</td>
</tr>
<tr>
<td>MI</td>
<td>25</td>
<td>CIN 1</td>
</tr>
<tr>
<td>MJ</td>
<td>20</td>
<td>CIN 1</td>
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<tr>
<td>MK</td>
<td>24</td>
<td>CIN 1</td>
</tr>
<tr>
<td>ML</td>
<td>25</td>
<td>CIN 1</td>
</tr>
<tr>
<td>MM</td>
<td>24</td>
<td>CIN 1</td>
</tr>
<tr>
<td>MN</td>
<td>25</td>
<td>No abnormality found</td>
</tr>
</tbody>
</table>
Plate 3.1

The protein product of pGEX2T (with no DNA insert) gives a single band when run on a 10% polyacrylamide gel but the fusion proteins produced by pGEX constructs including HPV16 genetic material (table 3.1) show multiple bands.

M: Protein size markers as indicated (kDa).

TG1: Whole bacterial lysate.

GST: Product of pGEX2T.

Other bands are the products of pGEX vectors with the HPV16 ORF indicated.

Predicted relative molecular weights of GST, 26kDa; of GSTE6, 43kDa; of GSTE7, 43kDa and of GSTL1, 60kDa.
Proliferation to fusion proteins.

_Proliferation assay method adaptations_

Most proliferation assays were carried out using freshly obtained PBMC without further manipulation. In an attempt to improve the specificity of the assay some experiments were conducted using as responder cells lymphocytes enriched for cells expressing CD4 and CD45RO (by an adherent step followed by depletion of CD8, CD19 and CD45RA using Dynabeads as described above), with mitomycin C treated adherent cells as APCs present at a ratio of 9 responder cells to 1 APC. However, although this gave a slightly greater amplitude of responses this was offset by the loss of cell numbers and the tendency for a large increase in background counts when using cells which had undergone several manipulations.

Early experiments used RPMI medium with 5% PCS, although this was sometimes satisfactory there was a tendency to produce high background counts in the absence of added antigen. Most experiments were therefore conducted using IMDM with 10% human serum which usually allowed a low background. Backgrounds sometimes varied enormously from day to day even with the same donor and the same batch of medium.

_Subjects tested_

PBMC were tested from three uncharacterised laboratory donors (LA, LF and LG) and one patient with a recent history of HPV16 positivity and treated CIN111 (WB).

_Results_

GST alone did not stimulate proliferation above background over a wide concentration range when incubated with PBMC from the single patient tested. In contrast three of the four HPV16 fusion proteins stimulated a clear response (figure 3.1). Aspects of this proliferation tested in a laboratory donor LG suggested that it was indeed a recall response. Proliferation peaked at around 5 days (data not shown)
and most of the proliferation was seen to be of CD45RO cells and not CD45RA cells (figure 3.2).

However, GST alone did stimulate proliferation from the three laboratory donors and reproducibility of results could not be maintained between batches of antigen (although they appeared identical on SDS PAGE). It was not possible to demonstrate whether the proliferation was caused by HPV proteins, GST or undefined contaminants in the antigen preparation.
Figure 3.1

Proliferation of PBMC from patient WB. The antigens (GST alone and GST fusion proteins) were added at the range of concentrations indicated to triplicate wells containing 1.8 x 10^5 PBMC. Each point is the mean of triplicates.

Although these results are highly significant statistically (all of the individual results with HPV16 fusion protein were greater than with GST alone) as described in the text they were not reproducible and the reason for the result is unclear.
Figure 3.2

A comparison of proliferation of non-adherent cells from donor LG with the addition of no antigen, standard control antigens (purified protein derivative of tuberculin - PPD and inactivated influenza virus - X31), GST, GST fusion proteins and a mitogen (PHA). Each bar represents the peak proliferation from a range of concentrations of antigen and the mean of triplicates at that concentration. For each antigen a comparison is made between the proliferation of whole non-adherent cells (white bars), cells enriched in CD4 and CD45RA (black bars - "naive") and cells enriched in CD4 and CD45RO (hatched bars - "memory"). The enrichments were made by depletion of CD8, CD19 and the appropriate unwanted CD45 antigen.

The pattern expected for recall antigens is shown by PPD and X31. There is a good response by whole non-adherent cells which is much decreased when responders are enriched in CD45RA ("naive") cells and amplified when responders are enriched in CD45RO ("memory") cells. The pattern is not seen in responses to the mitogen as would be expected. All of the experimental antigens show the same pattern as the control antigens suggesting that proliferation of responders to these antigens is a standard recall response.
Proliferation to peptides

Peptides were used singly or in groups at a wide range of concentrations. No peptide-specific proliferation was detected from any donor including all three of the patients who gave a positive response in the vaccinia assay discussed in chapter 4 (data not shown).

Discussion

Although many of the early experiments produced promising results, a recall response to HPV16 could not be demonstrated in the peripheral blood of any of the donors tested by using bacterial fusion proteins or synthetic peptides and these conventional methods. It is not possible to say from these results whether this is because of technical problems in producing the right antigens in sufficient purity, whether primed cells are simply not present in the peripheral blood or whether there is some other reason why these methods will not work in this system.

On reviewing the literature it is striking that there are no reports of work in which a simple, conventional proliferation assay has clearly detected reproducible HPV16-specific responses in the peripheral blood of human subjects. Those studies which have claimed to detect such responses have been forced to use complex methods in which cells are pre-incubated in the presence of added cytokines. In several of the studies the incubations were repeated twenty or more times and an individual with a certain proportion of responding lines was adjudged to be a "responder". As described in the introduction to this section even a simple positive proliferation assay would not unequivocally demonstrate the presence of primed CD4 T cells. These cautions apply to an even greater extent when the proliferation assay has been preceded by other steps in vitro.

In the interests of clarity in the following discussion the papers discussed will be cited once and then referred to by the name of the first author only.
The earliest published results were in 1989 (Cubie et al. 1989). HPV18 E6 and HPV16 E6 and E4 were all used as bacterial fusion proteins with the relevant fusion partner alone as a control. The subjects were patients with various degrees of CIN and uncharacterised female laboratory donors. No subjects were tested for HPV16 or 18 on the cervix. Although proliferation greater than controls was found in a small proportion of both groups there was considerable variation between the 5 replicate wells for each result and no response was ever detected earlier than 7 days after setting up the cultures.

In 1990 another study demonstrated proliferation of PBMC in response to HPV16 L1 peptides which had been chosen using an algorithm to find sequences which were similar to known T cell determinants. (McIndoe 1993, Strang et al. 1990). Four of their eight L1 peptides stimulated responses from some of their donors after a six day assay using PBMC. This was true for laboratory donors as well as women with CIN. When a set of truncated peptides were used in an attempt to define epitopes consistent responses were not obtained from PBMC. The rest of their studies were carried out with T cell lines and clones. The lines were generated by culturing PBMC with a pool containing all eight peptides for more than two weeks, with regular supplements of IL2, antigen and feeder cells. Lines and clones responded variably to truncated peptides and analogous peptides from different HPV types. Although tentative conclusions were drawn about T cell determinants and the possibility of cells cross reacting with other HPV types the possibility was accepted that all of these responses resulted from priming in vitro.

McIndoe extended this work to compare groups of patients with cervical cancer or with CIN to laboratory donors (McIndoe 1993). PBMC were used rather than T cell lines and there was tendency for a much greater amplitude and frequency of response in the patients than in the controls. Four peptides from HPV16 E6 were also tested. Responses to L1 and E6 peptides tended to correlate in individuals.
A similar method has been used with HPV16 E7 peptides (Altmann et al. 1992). Although proliferation of PBMC to several HPV16 fusion proteins was initially demonstrated in a five day assay for their two donors, all further work was done using T cell lines or clones which were generated using a set of overlapping 14mer peptides covering the whole of E7. Some of these lines responded to the same peptides individually or in smaller pools. Some clones also were cytolytic to autologous EBV-transformed B cells pulsed with peptide. The subjects were asymptomatic individuals identified as seropositive by testing sera on a Western blot with HPV16 E4 and E7 fusion proteins. It was concluded that recall responses to HPV16 had been demonstrated as well as the epitopes in E7 relevant for the HLA types of their two donors.

A much-cited study used nine overlapping peptides of between sixteen and twenty-six amino acids which covered the whole of E7 (Kadish et al. 1994). Responses of PBMC could not be demonstrated in five or six day assays with single peptides so the culture was extended up to twenty one days with supplements of peptide, feeder cells and IL2. The proliferation of these cells was assessed by pulsing aliquots with \[^{3}\text{H}]\text{TdR}\) on day 14 and day 21. T cell lines were not generated with pooled peptide. The subjects were women with CIN who were later tested for HPV on the cervix. Many of the subjects responded to several of the peptides and there was a tendency for women with documented infection with HPV16, 18, 31 or 33 to be more likely to show responses. One patient with documented HPV31 had multiple genital warts which regressed spontaneously, two weeks after the regression a peptide response was found. This result could not be repeated three months later.

A group at Guy's hospital used PBMC from women with CIN and from controls with normal cytology (Shepherd et al. 1996). Twenty T cell lines were generated from each individual using HPV16 L1 as a fusion protein and supplementing the culture with IL2 and IL4. These lines were then restimulated with peptide and proliferation was assessed after 3 days. The peptides covered about 40% of the L1 protein and
were 15mers overlapping by 5. A response of two or more of the twenty lines to a peptide was taken as positive. It was concluded that possible human T cell epitopes had been identified although HLA typing was not reported. In agreement with the previous studies, there was a tendency for women with HPV16 infection and CIN to be responders.

Subsequently a Dutch study used a method very similar to that of Shepherd et al (de Gruijl et al. 1996). PBMC were cultured for two weeks in the presence of HPV16 E7 fusion protein and IL2 was added after one week. The lines were restimulated by a pool containing 14 overlapping 20mers which covered the whole of E7. The original number of circulating HPV16-specific memory cells was taken to be indicated by the proportion of twenty lines which incorporated more than twice as much[^H]TdR as lines incubated with no antigen. 51 women with abnormal cervical cytology were studied. There was a tendency for patients with persisting HPV16 to give a greater response than those with no HPV16 or a previous transient infection. This group also tested for IgG reactivity to an HPV16 E7 peptide but found no correlation with proliferative responses.

A very large American study used the resources of a large cohort of HPV-typed women (Tsukui et al. 1996). This work did not look for proliferation but at IL2 concentration in the supernatant of PBMC cultured for seven days with pools of peptides. The peptides were overlapping sets of mostly 15mers covering the whole of HPV16 E6 and E7. The subjects were 115 women with various degrees of CIN or cervical cancer and a control group of 25 women who had at some time tested positive for HPV16 but not developed cytological abnormality. Responses to at least one of the pools was found in 35% of the controls 17% of women with high grade CIN and 7% of women with cancer. Approximately 50% of each group gave a positive response to inactivated influenza virus. It was claimed that these results showed that progression of HPV16 related CIN is associated with a lower probability of a response to HPV16 E6 or E7 proteins.
Measuring stimulation of lymphocytes by peptides is a long way away from assessing the response of an organism to a pathogen and many assumptions have to be made if it is to be claimed that peptide-stimulated lymphocytes have any biological relevance.

Even if it is clear that a peptide derived from a virus has triggered specific T cell activation through the TCR it does not follow that the host has previously been exposed to the virus. It has been established that naive T cells can sometimes be primed \textit{in vitro} using procedures which are much shorter and simpler than those used by Strang \textit{et al.}, Altmann \textit{et al.}, Kadish \textit{et al.}, and Shepherd \textit{et al.} (Young, Daser, and Beverley 1993). These responses (to complex antigens) peaked at day seven and were clearly detectable by day six. Nearly all of the reports of HPV16 recall responses detailed above required at least six days in culture and most of them required the prior establishment of T cell lines and a culture period of two to three weeks. Although the possibility of priming \textit{in vitro} is discussed by most of these authors they provide little evidence to refute it (although de Gruijl \textit{et al} do report that they found no responses using their assay on umbilical cord blood). There is no certain way of distinguishing these two sorts of responses using the relatively crude measure of $[^3\text{H}]\text{TdR}$ incorporation. It can be attempted by separating cells according to their expression of surface markers known to be associated with the naive or memory phenotype (as in figure 3.9). However it is possible that cells expressing CD45R0 (the memory phenotype) can sometimes revert to expression of CD45RA (the naive phenotype) as has been shown in rats (Bell and Sparshott 1990). Other measures which can be used include examining the time course of proliferation (primary responses tend to take longer), examining the precursor frequency of responding cells (memory cells may be at a higher frequency) and looking for responses in umbilical cord blood where one would expect nearly all lymphocytes to be unprimed.
The best approximation *in vitro* to natural antigen presentation would be achieved by using whole virions. This is not possible for HPV16. Of the remaining choices viral proteins are better than peptides. Peptides are effective in proliferation assays only when used at concentrations which are far higher than could possibly be achieved *in vivo* by the peptide component of the whole pathogen. This may mean that some peptide epitopes are successfully presented by binding to MHC class II on the surface of the APC when, because of constraints in processing, they could not have been presented naturally.

Many laboratories have produced HPV16 proteins by a variety of methods but there are only two reports of HPV16 specific proliferation of PBMC using a bacterial fusion protein in a short term assay. Firstly Cubie *et al.*, who found no responses before seven days and secondly Altmann *et al.* who reported specific proliferation from two donors in a five day assay using HPV16 E4, E6, E7 and L1. In spite of this the bulk of their work used T cell lines generated using pooled peptides and only one such line responded to the whole protein. Several of the reports outlined above which used T cell lines describe the lack of responses to fusion proteins after up to a week *in vitro*. This is often put down to a low frequency of responding cells.

The reports of responses by PBMC to HPV16 peptides in a short term assay are more variable. It is to be expected that frequencies of T cells primed to a single peptide will be much lower than to the whole protein. Strang *et al.*, and McIndoe used peptides only and measured proliferation at six days. Altmann *et al.*, Kadish *et al.*, and Shepherd *et al.* did not obtain short term proliferation to peptides. Tsukui *et al.* used pools of peptides for seven days and read out on IL2 concentration, the method they used was first shown to be successful at stimulating CD4 responses to peptides from HIV gp160 chosen as likely to be good class II binders (Clerici *et al.* 1989). It is notable that only Strange *et al.* and McIndoe used peptides specifically predicted to bind to class II, the other groups were depending on processing of overlapping peptides to produce the right sequences. When Strang *et al* attempted to find
responses to truncated versions of their peptides they found that short term assays were unsuccessful and had to resort to making T cell lines.

Although in principal it is a simple, although expensive, matter to produce a set of peptides which represent a larger protein, in practice there are limitations. For instance ten of the peptides produced by Tsukui et al were insoluble and so could not be used (Tsukui et al. 1996). As a result there was incomplete coverage of E6 and E7. Even when soluble peptides can be produced the optimal length to restimulate primed class II restricted human lymphocytes varies unpredictably with the epitope and the donor (Reece et al. 1994). Studying PBMC proliferation to epitopes from tetanus toxoid, Reece et al found that although responses to some epitopes can be stimulated by overlapping 12mers others require 14 or 16mers. In two of three donors responses to one epitope could only be detected by using a 21mer. By contrast 3 of 4 31mers including the whole of 4 known epitopes failed to stimulate proliferation of PBMC presumably because of limitations on processing. Worryingly they also found that the same donor tested with the same peptide pools after an interval of two weeks gave a substantially different pattern of results.

Finally, even if these findings are given the benefit of the doubt there is very little agreement between them. Although the Shepherd et al. peptides covered a region of L1 which contained two of the Stranger et al. peptides nearly all the positive responses came from elsewhere. Those papers which have attempted to draw tentative epidemiological conclusions have commented on whether detection of CD4 responses is more or less likely in patients with persisting HPV16 infection. McIndoe (E6 and L1), Shepherd et al. (L1), deGrujil et al. (E7) and Kadish et al. (E7) all found this to be more likely whereas Tsukui et al. (E6 and E7) found the opposite. (Tsukui et al deliberately chose as controls women who had had HPV16 infection in the past but not in more recent testing.)
In conclusion, taking my results together with the data reviewed above it is far from certain that HPV16 primed CD4 cells have yet been detected in the peripheral blood. Possible reasons for this will be discussed further in chapter 6.
Chapter 4

Detection of memory CD4 lymphocytes specific for HPV16 in humans using recombinant vaccinia
Background

As described in chapter 3 it has proved very hard to demonstrate primed CD4 T cells in peripheral blood by conventional means. It has been shown that vaccinia-specific proliferation of lymphocytes can be stimulated by culturing PBMC with live vaccinia preparations (Littaua et al. 1992). This is a simple and reproducible method. The work in this chapter was carried out to see whether live recombinant vaccinia when cultured with PBMC could stimulate proliferation of lymphocytes primed by natural infection with HPV16.

Vaccinia virology

Classification

Vaccinia is a member of the family Poxviridae and the genus Orthopoxvirus. These antigenically related viruses are cross-protective and include variola - the smallpox virus and cowpox - the virus originally used by Edward Jenner in his pioneering vaccinations against smallpox (Jenner 1798). The origin of vaccinia remains unclear and there is no natural host, it is speculated that it could have derived from mutation of cowpox (Fenner et al. 1988). There is evidence that it has been used for vaccination at least since 1876 (Fenner et al. 1988).

Since the eradication of smallpox, the only poxviruses known to infect humans are molluscum contagiosum and rare zoonoses such as cowpox, monkeypox and orf.

Morphology, cell entry and replication

Poxviruses have large and complex virions. Vaccinia virions measure about 350 by 270 nm and occur in two morphologically distinct forms: the intracellular mature virions (IMV) and the extracellular enveloped virions (EEV). EEV is the form which is released from the cell, it has an extra lipid envelope and 10 associated proteins
which are absent from IMV. The virions contain a linear double-stranded DNA genome of about 200kb and numerous polypeptides (Moss 1996).

Poxviruses replicate in cytoplasmic "factories" within the infected cell. The virus encodes all the enzymes necessary for DNA replication and transcription.

Vaccinia, in common with cowpox and monkeypox, has a wide host range and most tissue culture cell lines are able to support replication. It is not known if there is a specific cell surface receptor although some antibodies to the virion are able to block entry into cells.

Gene expression and DNA replication.

Gene expression is regulated by control of transcription and the viral genes are classified as early, intermediate or late with peak mRNA levels detectable at about 1 hour, 2 hours and 4 hours respectively. The virion contains enzymes capable of transcribing the early genes immediately on entry into the cell. DNA replication starts about 2 hours after infection (Baldrick and Moss 1993).

Virion production

The membranes enclosing the infectious IMV virions are thought from EM evidence to come from the intermediate compartment between the endoplasmic reticulum and the Golgi stacks (Sodeik et al. 1993). Some of the IMV then acquire a further double layer of membrane and migrate to the cell surface where the outer membrane fuses with the plasma membrane releasing the EEV from the cell. However for the Western Reserve strain used in this work most EEV remains associated with the cell surface (Blasco and Moss 1992).

Recombinant vaccinia

As much as 25000 bp of DNA can be added to the genome of vaccinia without the need for deletions (Moss 1991). Recombinant vaccinia has been widely used to
deliver antigen to the cytoplasm of antigen presenting cells where it can be processed and loaded onto class I MHC [reviewed in Bennink, 1990 #114]. Vaccinia-specific proliferation of lymphocytes can be stimulated by culturing PBMC with either live or UV-inactivated vaccinia preparations (Littaua et al. 1992). Although loading of MHC class II for presentation of antigen to CD4 lymphocytes is usually thought to require exogenous antigen, it has been well documented that antigens encoded by recombinant vaccinia can enter the class II processing pathway and activate class II restricted T cell clones (Jaraquemada, Marti, and Long 1990, Lee et al. 1993, Malnati et al. 1992). Indeed there is evidence that some class II restricted determinants are presented preferentially if synthesized within the APC perhaps because they are unusually labile (Eisenlohr and Hackett 1989).

Methods

Proliferation assay with vaccinia recombinants

The assays were set up exactly as the PBMC proliferation assays described in the method section of chapter 3 but with serial dilutions of vaccinia stocks prepared by the methods below. Stocks were of titre between $5 \times 10^8$ and $5 \times 10^9$ plaque forming units per ml. The dilution range was chosen from pilot experiments to include concentrations both higher and lower than that which causes maximal proliferation (see for example figure 4.1). The result of these assays is routinely expressed as the maximal proliferation obtained from this range of dilutions, so, for instance, the result shown in full in figure 4.1 is also represented by the bars in figure 4.2 (subjects LE and LC).

Maintenance of cell lines

Adherent cell lines: CVI and TK$^-$ 143

These cell lines were maintained in E4 medium with 5% FCS. When confluent the monolayer was washed with versene (PBS with 0.5mM EDTA) and incubated in
trypsin/versene (versene with 0.05% w/v trypsin) until the cells were detached (5 minutes for TK-143, 10-15 minutes for CV1). They were then washed with E4 and reseeded at the appropriate dilution.

**Mycoplasma testing**

Cells were tested for mycoplasma by growth of colonies on agar (Cell services ICRF).

**Vaccinia viruses**

Source of vaccinia viruses

All vaccinia used in this research was of strain Western Reserve. All of the recombinants were gifts from other workers. Inserts used were as follows: no insert: VVWT, murine cyclin D1: VVCd1 (Dahl, Beverley, and Stauss 1996), influenza nucleoprotein: VVNP (Cerundolo et al. 1995), HPV16 E6: VVE6 (Gao et al. 1994), HPV16 E7: VVE7 (McLean et al. 1993), HPV16 L1: VVL1 (Zhou et al. 1991a). VVE6 and VVL1 are mycophenolic acid resistant constructs. All other inserts are in the thymidine kinase gene.

**Production of TK- vaccinia virus stocks**

TK- recombinants (VVCd1, VVE6, VVE7, VVNP) were grown in TK- 143 cells under selection with BrdU. TK- 143 cells were grown to about 80% confluence and infected with vaccinia at more than 3 pfu/cell (crude vaccinia lysate was diluted into the minimum volume of E4 medium to cover the monolayer, this was incubated for 2 hours with frequent tipping) E4 with 5% FCS and 10μg/ml BrdU was then added and the flasks kept in a CO2 incubator for 48 hours. The cells were then scraped off the flask using a cell scraper, centrifuged, resuspended in a small volume of E4 and lysed by rapid freeze/thawing before being dispersed by mild sonication. This crude lysate was frozen in aliquots and assayed for plaque-forming activity.
Production of MPA resistant vaccinia virus stocks

MPA resistant recombinants (VVL1 and VVE6) were grown in CV1 cells under selection with MPA, xanthine and hypoxanthine. The procedure was identical to that for TK⁻ recombinants except that the medium used for the 48 hour incubation was E4 with 5% FCS, MPA, xanthine and hypoxanthine.

Production of wild type vaccinia virus

Wild type virus (VVWT) stocks were grown on TK⁻143 cells as for recombinants but without BrdU selection.

Plaque assay

TK⁻ 143 cells or CV1 cells were seeded into 6 well plates and grown overnight to approximate confluence. The wells were washed with PBSA and serial 10 fold dilutions of the vaccinia recombinant stock were added at 250μl/well. The plates were incubated for 2 hours with frequent tipping and then overlaid with the appropriate selection medium (as for production of stocks). After a further 48 hours of incubation the wells were washed with PBSA and the monolayers were stained with 0.1% w/v crystal violet in 20% v/v ethanol. Plaques could then be visualised and counted and the titre of stocks calculated.

Ultra violet light inactivated vaccinia

Two aliquots of virus were made at the highest dilution. One was then irradiated with short wave ultra violet light from 5cm for 15 minutes. Further dilutions were made from both aliquots. Ultra violet light-irradiated virus was unable to form plaques in a standard assay.
Results

PBMC from individuals who had received smallpox vaccination proliferated vigorously in the presence of live vaccinia whereas PBMC from unvaccinated individuals proliferated much less, if at all (figure 4.1). The amount of cellular proliferation was sensitive to small changes in the concentration of vaccinia added. Peak responses corresponded to a multiplicity of infection of about 1 for each of the vaccinia constructs studied. (The multiplicity of infection is the ratio of the number of plaque-forming units of virus to the number of cells in a well.) At higher concentrations proliferation was below background but it rose sharply as vaccinia concentration fell so that a ten fold change in concentration was sometimes sufficient to go from background levels to maximal response. Further dilution of vaccinia resulted in a more gradual decline in proliferation to background levels. Because of the great sensitivity of the assay to the concentration of virus (and the imprecision of the plaque forming assay to determine the concentration of viral stocks) all assays were performed with a range of dilutions of vaccinia constructs, but for simplicity the result is generally expressed as the maximal proliferation. So for instance the data shown in figure 4.1 are simplified into LE and LC in figure 4.2.

For reference all of the results using the vaccinia assay on blood from laboratory donors or patients are shown in full in the appendix.
Laboratory donors: vaccinated and unvaccinated

Laboratory donors comprise three groups. Definitely received smallpox vaccine (n=5), definitely not vaccinated (n=7), uncertain of vaccination status (n=3). In order to control for the possibility of responses to vaccinia itself all assays included the two control viruses VVWT and VVCd1. All responses to control viruses from donors known to have been vaccinated were greater than those from donors not known to have been vaccinated (figure 4.2). The highest amplitude response was from a donor (LL), uncertain of her vaccination status, who had definitely not received vaccination for at least 20 years. One unvaccinated laboratory donor made responses to VVL1 and to VVNP without responding to any of the other constructs (figure 4.3). PBMC from this individual were tested 6 times over a period of 26 months. During this time responses to VVWT, VVCd1, VVE6 and VVE7 remained consistently low, however after a gap of 17 months in testing a response to VVL1 was detected which gradually declined. Although it was detected in three separate assays over a period of five weeks it could not be detected 7 weeks later (figure 4.4).
Figure 4.1

Proliferation of PBMC from two individuals after five days in culture with dilutions of live vaccinia constructs. LC had received recent vaccinia vaccination, LE had never been vaccinated. As well as showing the critical dependence of proliferation on the concentration of virus this figure shows the way in which all of the proliferation data in this chapter was obtained. These results, for convenience, can also be shown as the maximal proliferation from the whole range of dilutions. In this form they appear as part of figure 4.2. For reference all of the results discussed in this chapter are shown in full in the appendix. In all of these experiments each point was performed in triplicate and any experiment in which the standard deviation of triplicates exceeded 15% of the mean were discarded (this was unusual).
Figure 4.2

Proliferation of PBMC from vaccinated and unvaccinated laboratory donors. The maximal result is shown from a range of dilutions of virus stock. Background proliferation of PBMC with no added antigen has been subtracted. This summary is extracted from the full results which are shown in the appendix.
Figure 4.3

Proliferation of PBMC from two donors to all of the vaccinia constructs. In each case the maximal proliferation is shown from a range of dilutions. The results for LE are also shown in figure 4.4 as week 0 (the full results are in the appendix).
Figure 4.4

Proliferative responses of PBMC from a single donor (LE) repeated 4 times in a 12 week period. Two identical experiments 17 and 22 months before this sequence gave negative results for all constructs. (Full results are in the appendix)
Results from HPV16 +ve patients

Twenty seven patients were tested (tables 3.1 and 3.2 and figure 4.5). Most patients were uncertain of their smallpox vaccination status. It is clear that if an individual responded to vaccinia because of smallpox vaccination any additional HPV16 response was masked. Once this problem became apparent the protocol was altered to exclude patients born before 1970 - routine infant immunisation against smallpox ended in the UK in 1971 (Cox, McCarthy, and Millar 1974). With a single exception, a positive response to VVWT and VVCD1 was always accompanied by a positive response to the HPV16 constructs (the exception was subject WA in figure 4.5 who gave a response to VVWT only at the highest concentration) so it was assumed that proliferation stimulated by VVWT or VVCD1 of greater than 5000 cpm above background was caused by responses to vaccinia itself (individuals known not to have been vaccinated never gave these responses). Fourteen results were thus eliminated. Of the remaining thirteen patients three (21%) gave responses to two or more of the HPV16 constructs which were clearly greater than to the controls (WB, WC and WF in figure 4.6). If a much more stringent cut off is set and all results with proliferation to controls of greater than 1500 cpm above background are eliminated eleven results remain (as can be seen in figure 4.6) including the three HPV16 responders (27%). These three individuals responded most vigorously to E6 or E7 but two of the three also responded to L1.

Assays were set up using dilutions of lysed TK-143 cells to check that the background proliferation was not caused by other components of the crude vaccinia stocks. Although very high concentrations of this preparation did stimulate some proliferation, dilutions of 1/2000 or more (the highest concentration necessary to achieve a multiplicity of infection of 1 using the most dilute viral stock) did not stimulate proliferation greater than that seen with no antigen (figure 4.7) most experiments used much greater dilutions.
Figure 4.5

An overview of the results from the HPV16-positive patients. Maximum proliferation of PBMC is shown from a range of viral dilutions - the backgrounds have been subtracted. These results are shown in detail in the appendix.
Figure 4.6

All patient results as in figure 4.5 but with subjects who gave a response of 5000 cpm or more to VVWT or VVCd1 eliminated. The first three patients in this figure (WB, WC and WF) appear to give positive responses to the HPV16 constructs. If, as suggested in the text, the cut-off is set at 1500cpm instead of 5000 a further three subjects are eliminated (WE, WL and MA).
Figure 4.7

Proliferation of PBMC from donor LG cultured with dilutions of cell lysate from TK-143 cells for five days. This result demonstrates that responses to vaccinia constructs were not caused by non-specific contamination from the cell lysate in which they were prepared. (A dilution factor of between 2000 and 20000 was required to achieve a multiplicity of infection of 1 in the proliferation assays)
For one of the three strong responders (WF), the assay was also set up after PBMC had been depleted of cells expressing either CD4 or CD8 (figure 4.8). After CD8 depletion the amplitude of the HPV 16 responses was much greater but the amplitude of the control responses was unchanged. CD4 depletion reduced the amplitude of all responses. PPD was included as a control in this assay and responses to this were amplified in the same way by CD8 depletion.

Reproducibility of results

Reproducibility of results was first established with laboratory donors. The pattern of responses to control viruses was maintained over periods of up to two years, however the responses to the HPV16 constructs was seen to vary as described above for donor LE (figures 4.3 and 4.4). Problems in recalling patients meant that only one of the three responding patients could be retested. 7 months after the initial result the HPV16-specific responses were not maintained (see figure 4.9 and results for WC in the appendix).

Ultra violet light -inactivated vaccinia

To test whether responses were simply due to preformed antigen in the vaccinia stocks the responses of a recently vaccinated donor to live and ultra violet light inactivated virus were compared across the same dilution range. The dilution of viral stock required to give maximal proliferation was not altered by ultra violet irradiation but the magnitude of the response was reduced by about 50% (figure 4.10).

Responses to VVNP

In order to test whether recombinant vaccinia virus was capable of revealing responses to well established recall antigens, the response to influenza virus nucleoprotein was tested. 3 laboratory donors and one patient were tested LM and
LE gave a clear response to VVNP compared with control vaccinia virus LN and MN did not. All gave a good response to whole inactivated influenza virus (figure 4.11). These results are shown in full in the appendix.
Figure 4.8

Proliferative responses of PBMC, CD4-depleted PBMC and CD8-depleted PBMC from WF- a HPV16-infected donor. The maximal result is shown from a range of dilutions of virus stock. All assays were performed simultaneously using the same cell number and virus dilution.
Figure 4.9

Proliferation of PBMC from donor WC (shown in figure 4.6) repeated after 7 months.
Figure 4.10

Proliferative responses of PBMC from a single vaccinia-vaccinated donor (LG). The same dilutions of wild type virus were used either live (VVWT) or after inactivation with ultra violet light (VVWT(UV)).
Figure 4.11

Proliferation of PBMC from three laboratory donors in response to all of the vaccinia constructs including VVNP. All three donors responded to whole inactivated influenza virus (X31). All results apart from X31 are the maximal from a range of viral dilutions as shown in full in the appendix. X31 was used at a previously established optimum dilution.
Time course of proliferation for a vaccinia vaccinated donor

All the constructs tested stimulated proliferation which was maximal after 4 to 8 days in culture. The control viruses: VVWT and VVCd1 caused peak proliferation at day 7 over a 16-fold dilution range. For the HPV E6 and E7 constructs there was a close correlation between time to maximal proliferation and dose of infecting virus. Higher doses of virus led to an earlier peak in proliferation and the maximum proliferation was only about half that of the control viruses (figure 4.12).

Viability of PBMC (as assessed by trypan blue exclusion) was greater than 90% even after 8 days in culture with live vaccinia.

Suppression of proliferation caused by VVE7 after prolonged culture

The decline in responses to VVE7 after 5 or 6 days in culture was investigated further. The phenomenon was reproducible even when responder cells were exposed to this antigen only on allogeneic adherent cells which had been pulsed with VVE7 for 2 hours at a multiplicity of infection of more than 5 then irradiated (45Gy) and thoroughly washed. After 8 days in culture such cells incorporated less of a pulse of DNA than control cells cultured with no antigen. Furthermore supernatants taken from such cells and filtered through a 0.2mm filter appeared to strongly inhibit proliferation of fresh PBMC stimulated with recall antigens but not those stimulated with mitogen. This effect was not always seen but when it occurred it was very powerful. The effect could also be seen under light microscopy as a diminution of the number and size of clumps of proliferating cells in the presence of the inhibitory supernatant. The effect could not be reproduced with any of the other vaccinia constructs (figure 4.13). It was eventually found that the supernatants obtained from VVE7-infected cells contained high levels of mycoplasma which was contaminating the stock of VVE7. Mycoplasma could not be detected by testing the supernatant directly but if mycoplasma-free cells were cultured with the supernatant for 7 days
Figure 4.12

Proliferative responses of PBMC from a single vaccinia-vaccinated donor (LG). A range of dilutions of virus were used at each time point and the maximum is shown.
Figure 4.13

Five day proliferation of PPD-stimulated PBMC cultured with medium only (control) or with dilutions of cellular supernatants obtained as described in the text. The vaccinia construct indicated was used in the preparation of the respective supernatant.
high levels were then present. When fresh stocks of mycoplasma-free VVE7 were used the supernatants obtained in the same way did not have this suppressive effect.

Discussion

This work has demonstrated that it is possible to detect antigen specific proliferation of lymphocytes by culturing PBMC with live recombinant vaccinia which expresses the antigen. This method was used to detect HPV16-specific proliferation in the peripheral blood of two laboratory donors and three patients infected with HPV16. In the patients, all of whom had biopsy proven CIN, responses were chiefly to E6 and E7 whereas the laboratory donors made responses to L1 only.

In addition, vaccinia-specific proliferation of PBMC was detected more than 20 years after smallpox vaccination. This is consistent with the work of Demkowicz et al who were able to generate CTL from the peripheral blood of similar subjects (Demkowicz et al. 1996).

The demonstration that depletion of CD8 cells amplified the response to HPV16 constructs without altering the response to VVWT or VVCd1 supports the contention that the proliferation is HPV16-specific and that most of the responder cells are CD4 T cells. That the method can detect recall responses is supported by the finding that responses to influenza nucleoprotein can be revealed with VVNP in some donors who respond to influenza virus itself. Presumably donors who respond to influenza virus but not VVNP are responding to other influenza virus proteins.

Live virus stimulated twice as much PBMC proliferation from a smallpox-vaccinated donor as ultra violet inactivated virus at the same dilution. This suggests that \textit{de novo} synthesis of antigen in the cytoplasm of the APC can increase the stimulation of responder cells without the inhibitory effect seen with increased extracellular
antigen. This mechanism could account for the power of the method to detect responses of HPV16-specific T cells which are likely to be present at low frequency.

It seems likely that one reason why it is hard to detect lymphocytes primed to HPV16 is because they are only present transiently in the peripheral blood. Both the individuals with positive results who were retested eventually became negative. Papillomaviruses cause a very localised infection confined to the epithelium and it is likely that the best place to seek primed CD4 T cells would be in the lymph nodes draining a resolving lesion which, of course, is not possible in humans.

This study and that of Demkovicz et al (Demkowicz et al. 1996) have demonstrated recall responses specific for vaccinia in the PBMC of individuals who have not been vaccinated for at least 20 years. This is an impressive demonstration of the length of T cell memory to a lytic virus which does not persist. Vaccination of infants did not confer lifelong protection from smallpox presumably because the rapidity of disease progression did not allow time for a full recall response to develop. It did however often attenuate adult infection (Fenner et al. 1988) and the longevity of vaccinia primed T cells probably accounts for the impaired response to a vaccinia virus vaccine expressing HIV gp160 seen in recipients who had received childhood smallpox vaccination (Cooney et al. 1991).

Although the responses of unvaccinated laboratory donors to all viruses were lower than those of vaccinated donors several were well above background. In addition a surprisingly high proportion of the patients (14 out of 27) gave quite high responses to VVWT and VVCd1 as well as to the other constructs. These responses were much commoner in subjects recruited from the clinic for sexually transmitted disease than in the gynaecology clinic patients. Although some of these undoubtedly had received smallpox vaccine it is unlikely that they all had. By 1969 only 35% of children born in England were vaccinated as infants and routine vaccination except for overseas
travel was withdrawn in 1971 (Cox, McCarthy, and Millar 1974). It is tempting to speculate that some of the vaccinia responses were primed by a different pox virus.

Although zoonoses such as cowpox and monkeypox are rare another pox virus, molluscum contagiosum, causes an infection which is common in childhood and as a sexually transmitted disease in young adults. It causes small discrete skin lesions which often resolve promptly if pricked with a needle - presumably through an immune response. What used to be merely a nuisance has become a considerable clinical problem for many patients with HIV-induced immune deficiency. The natural history is quite different from that of the Orthopoxviruses and there clearly must be major differences in viral structure and organisation. It is not surprising that there is no serological cross protection. However there are regions of similarity in these large, complex viruses. Molluscum contagiosum has recently been fully sequenced: 104 predicted proteins have counterparts in vaccinia with up to 76% homology (Senkevich et al. 1996). Patients attending a clinic for sexually transmitted disease may be quite likely to have generated an immune response to this virus which can perhaps be recalled by exposure to vaccinia antigens in vitro. Conversely, if lymphocytes primed by vaccinia during smallpox vaccination were restimulated by cross reactive molluscum contagiosum antigens this could account for the observed longevity of some vaccinia responses.
Mycoplasma contamination

Mycoplasma is notorious for the difficulties it causes when infecting cultured cells, it is capable of passing through 0.2mm filters which will exclude most microorganisms and has a variety of effects on lymphocytes and antigen presenting cells including activation of macrophages (Bertoni et al. 1978), stimulation of interferon production (Birke et al. 1981) and mitogenic effects (Naot et al. 1979). The "suppressive" effects on cells infected with VVE7 and filtered supernatants from these cells were most likely to have been caused by mycoplasma contamination of the TK-143 cells used to grow the particular batch of vaccinia. Although mycoplasma could not be detected in cell-free supernatant this reflects the limitations of routine methods of mycoplasma detection and the fact that the organism needs cells to be present in order to replicate. The effect of this low level contamination was not seen until after several days in culture which explains why it failed to "suppress" 2 day mitogenic stimulation of PBMC. When fresh stocks of VVE7 free of mycoplasma were used the "suppressive" effects could not be reproduced. It is likely that mycoplasma-associated thymidine phosphorylase caused rapid degradation of \[^{3}H\]TdR so that it was not available for incorporation into stimulated cells (Merkenschlager et al. 1988a) although this was not formally demonstrated.

The reason for the inhibition of PBMC proliferation seen after more than 6 days in culture with VVE6 or VVE7 is not clear. Although the initial observation with VVE7 appears to have been an artefact caused by mycoplasma contamination, the same observation was also made using VVE6 which has been consistently clear of mycoplasma infection. It remains tempting to speculate that it is caused by a toxic or suppressive action E6 or E7 on cells of the immune system and that there may be a correlate of this in vivo which could account both for the immune evasion of HPV16 and for the practical difficulties universally encountered in detecting primed T cells in humans. In support of this speculation are anecdotal accounts of difficulties
encountered in establishing and maintaining cell lines transfected with DNA encoding these proteins and the observations in chapter 4 of the response of PBMC to E6 and E7 as fusion proteins.

The method described has proved useful and reproducible but uses simple observations from a very complex system. Vaccinia viruses have large complex genomes and employ a variety of mechanisms to evade immune responses many of which have been described (Smith 1993). It is quite likely that vaccinia-encoded immunomodulatory proteins influence these proliferation assays (as of course they may influence CTL assays many of which routinely use recombinant vaccinia). PBMC are a complex mixture of cell types and proliferation assays themselves while useful and informative must be interpreted with caution. Incorporation of $[^3]$H]TdR does not always closely reflect cellular proliferation (Ashman 1984) and cellular proliferation may not closely reflect specific T cell activation. It would be unwise to suggest that the reasons why these assays work is completely understood.

In summary a novel approach has been described to detecting primed CD4 T cell responses in the peripheral blood. The method has proved capable of detecting HPV16 specific responses of cells which seem to be present only transiently in the blood.
Chapter 5

Detection of CD4 memory lymphocyte responses to HPV16 in mice
Aims

As explained in chapter 4 it has proved very difficult to demonstrate human class II restricted lymphocytes primed to HPV16. A different approach to examining lymphocytes restricted by human class II is to prime mice which are transgenic for DR or DQ (Schwarz 1994).

The aim of the experiments described in this chapter was to establish a method which could consistently demonstrate a CD4 proliferative response to HPV16 in mice so that a comparison could be made with the responses of mice transgenic for human MHC class II.

Methods

Recombinant DNA methods

Preparation of plasmid DNA

Bacterial stocks containing plasmids were stored at -70°C with 15% (v/v) glycerol. A scrape taken from the stock was inoculated into 10 ml LB medium with appropriate antibiotics. This was incubated with agitation at 37°C for at least 6 hours and usually overnight. If a larger volume was required this culture was added to more LB with antibiotics and again incubated with agitation. Bacteria were harvested by centrifugation at 2500 x g for 30 mins. DNA was prepared using kits supplied by QIAGEN GmbH according to the manufacturer's instructions.

Restriction digest of DNA

Restriction enzyme digests were carried out in volumes of 10-100 µl with a 10-20 fold excess of enzyme. "One phor all" buffer (Pharmacia) was used at the optimum concentration recommended by the supplier.
Restriction enzymes, polymerases and ligases

Please see chapter 2

Quantitation and storage of DNA

DNA was obtained in TE buffer at pH8, unless otherwise described, and stored at -20°C. Stocks were quantified by measuring absorbance at 260 nm (assuming that 50µg/ml DNA gives OD\_260 = 1).

Ligation of DNA and transformation of competent cells

Where necessary DNA fragments were "blunted" using Klenow fragment of E. coli DNA PolI or T4 DNA polymerase. Ligation was achieved with high concentration DNA ligase (Gibco). Transformation of competent cells (Epicurian coli, Stratagene) was accomplished by electroporation.

Agarose gel electrophoresis and recovery of DNA from agarose gels

Agarose was dissolved in TE buffer by boiling, the solution was allowed to cool to about 50°C and ethidium bromide was added to give a final concentration of 0.5 µg/ml. Gels were poured in plastic trays and run at 90 volts until sufficient separation was obtained. DNA was visualised with long wave ultraviolet light. The size of fragments was estimated with a 1 kb ladder (Gibco).

DNA was purified from agarose gels by cutting out the appropriate band under UV illumination, dissolving the agarose and extracting the DNA using a QIAEX II kit (Qiagen GmbH) according to the manufacturers instructions.
**DNA sequencing**

This was performed on an ABI PRISM 377 DNA sequencer (Perkin Elmer, ICRF DNA sequencing service) samples were prepared by cycle sequencing using dye labelled terminators.

**Mice**

Mice were obtained from Biological Services, Clare Hall Laboratories ICRF. Unless specifically mentioned they were female, C57Bl6, aged between 10 and 20 weeks at the start of an experiment. They were kept in the animal facilities at the Biological Services Unit, University College London. Experiments involving live vaccinia were conducted inside the level 2 containment facility at the same unit. Mice were caged with between 2 and 6 mice per cage.

**Immunisation of mice**

For immunisation with complete Freund's adjuvant antigen was mixed with an equal volume of adjuvant and briefly sonicated using a sonifier 250 (Branson) to create an emulsion which was used immediately.

**Preparation of cell suspensions from murine spleen or lymph nodes**

Mice were killed and immediately dissected to remove the spleen or appropriate draining lymph nodes. For mice primed with protein in the footpad popliteal and inguinal nodes were used. For mice primed with protein at the base of tail, or with DNA in the hind leg, para-aortic and inguinal nodes were used. The nodes or spleen were transported in IMDM or RPMI medium on ice and as soon as possible a single cell suspension was made by forcing the tissue through a sterile plastic mesh tea strainer with the plunger from a 5ml syringe. The cells were immediately washed and resuspended in the final medium.
Methods and results with protein antigens

Antigens used for priming mice

The HPV16 antigens used were: recombinant vaccinia with HPV16 E7, recombinant vaccinia with HPV16 L1, HPV16 L1 purified from yeast cells, HPV16 E7 GST fusion protein purified from bacteria and peptides from HPV16 L1 and E7 used individually or in pools (Peptide sequences are detailed in fig 2.1, 2.2 and 2.3).

The other antigens used for establishing a working method were KLH and ovalbumin.

Methods for priming mice

Because it proved hard to find any responses at all a variety of routes, methods and timings were used, these are outlined in table 5.1.

PHA consistently stimulated proliferation in vitro. In one set of experiments mice were primed with VVE7 and strong proliferation of splenocytes was stimulated by GST E7 but not GST alone. However GST E7 and GST E6 also stimulated splenocytes from unprimed mice presumably because of impurities in this batch of the bacterial fusion protein (figure 5.1). In all other experiments mice primed with VVE7 responded to VVWT in vitro but not to GSTE7 or to E7 peptides (figure 5.2).

In order to ensure that the lack of response was not caused by faulty reagents or experimental technique mice were primed with the well documented antigens KLH and ovalbumin (Sercarz et al. 1993). These were injected at the base of tail after emulsification with complete Freund's adjuvant. Draining lymph nodes (para-aortic and inguinal) were obtained 7-10 days later. Recall proliferation was seen using these antigens and the timing and conditions used in vitro were then optimised as described below.
### Table 5.1 (continued on next page)

**Overview of mouse experiments**

<table>
<thead>
<tr>
<th>Priming</th>
<th>Boost</th>
<th>In vitro †</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>VVE7 (1.5x10⁷ pfu) Intraperitoneal</td>
<td>Day 10</td>
<td>Day 20-48</td>
<td>VVWT response only (see figure 5.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spleen cells with peptides, GST fusion protein or VVWT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(As priming)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
<td>Day 28</td>
<td>No response</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spleen cells with either peptide, VVWT or VVE7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(As priming)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/ E7 peptide 34-58</td>
<td></td>
<td>Day 7-10</td>
<td>Positive response with each (but naive mice responded to KLH) figures 5.5 and 5.6</td>
</tr>
<tr>
<td>2/ E7 peptide 45-69</td>
<td></td>
<td>Cells from draining lymph nodes with same antigen</td>
<td></td>
</tr>
<tr>
<td>subcutaneous injection with IFA* (50μl of 0.1 mM peptide)</td>
<td>No</td>
<td>Day 7-10</td>
<td>Positive response with each (but naive mice responded to KLH) figures 5.5 and 5.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cells from draining lymph nodes with L1 and E7 peptides</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>Day 7-10</td>
<td>No response</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cells from draining lymph nodes with L1 and E7 peptides</td>
<td></td>
</tr>
</tbody>
</table>

† Cells responded to PHA in all assays, * IFA: incomplete Freund's adjuvant, # CFA: complete Freund's adjuvant
Table 5.1 (continued)

<table>
<thead>
<tr>
<th>Priming</th>
<th>Boost</th>
<th>In vitro †</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>VVL1 (2.5x10⁷pfu) intravenously</td>
<td>Day 21: VVL1 (2.5x10⁷pfu) base of tail with CFA</td>
<td>Day 29 Cells from draining nodes with L1 peptides</td>
<td>No response</td>
</tr>
<tr>
<td>VVL1 (2.5x10⁷pfu) base of tail with CFA</td>
<td>No</td>
<td>Day 7-10 Cells from draining nodes with L1 peptides</td>
<td>No response</td>
</tr>
<tr>
<td>L1 peptides or E7 peptides in pools or singly, 100µg of each peptide in base of tail with CFA#</td>
<td>No</td>
<td>Day 7-10 Cells from draining nodes with various peptides singly</td>
<td>Responses to peptides shown in figures 5.8 and 5.9</td>
</tr>
<tr>
<td>pCDNA3.16E7 or pCDNA3.16L1, 50µg DNA in water, intramuscular injection in both hind legs</td>
<td>One or two boosts after at least 28 days (as priming)</td>
<td>Day 7-10 Cells from draining lymph nodes with VVE7, VVL1, all peptides singly, L1 VLP</td>
<td>Responses of L1-primed mice to L1 VLP only (figure 5.10)</td>
</tr>
</tbody>
</table>

† Cells responded to PHA in all assays, * IFA: incomplete Freund's adjuvant, # CFA: complete Freund's adjuvant
Figure 5.1

Proliferation of pooled spleen cells removed on day 21 from two mice immunised with 1.5 x 10^7 pfu VVE7 on day 1 and day 10. The four columns for each antigen represent concentrations of 50, 12.5, 3.1, and 0.75 mg/ml, highest concentration on the left. Results given as the mean of triplicate wells. HL1 medium used in vitro.
Figure 5.2

Proliferation of pooled spleen cells in response to WTVV (see table 5.1). The spleen cells were removed on day 28 from two mice immunised with $1.5 \times 10^7$ pfu VVE7 on day 1 and day 10. HL1 medium was used \textit{in vitro}. The results are given as the mean of triplicate wells. The bars include the highest and lowest of the triplicates.
However it was then clearly established that KLH could also stimulate proliferation of lymph node cells from mice primed with a different antigen (figure 5.3). This was not seen with ovalbumin.

*Methods used in vitro*

Initial experiments used HL1 serum free medium (Altmann et al. 1995) this had the advantage of low background proliferation of cells without antigen but also relatively low levels of proliferation even with mitogens. In contrast both IMDM with 10% FCS and RPMI with 0.5% normal mouse serum stimulated very high background proliferation. Eventually consistent results were obtained by using IMDM with a particular batch of FCS.

Cells were incubated for between 2 and 5 days before $[^{3}\text{H}]	ext{TdR}$ was added for the last 6-16 hours. Both round and flat-bottomed wells were tried. The best results were obtained after 4 days in flat-bottomed plates.

Experiments were conducted with and without cells added to act as APCs. These were irradiated whole splenocytes or irradiated, T- depleted splenocytes. Increases in proliferation which APCs allowed were offset by increases in background proliferation.
Figure 5.3

Proliferation of pooled lymph node cells taken at day 8 from mice primed with GST E7 in complete Freund's adjuvant in the base of the tail. KLH was at 100 μg/ml, only one representative peptide result is shown but all the E7 peptides were used at concentrations of 30 and 3 μg/ml and gave similar negative results. Medium was IMDM with 10% FCS. PHA (mitogen) stimulated 200,000 counts per minute. Results are the mean of triplicates.
Results using optimal method

In no experiment did peptides stimulate proliferation of cells primed with HPV16 antigen from another source (table 5.1)

However two of the L1 peptides consistently stimulated proliferation *in vitro* when the same peptide alone or as part of a mixture of peptides had been used to prime mice. These peptides were 86-110 and 305-329 (figure 5.4). None of the other L1 peptides did this.

None of the E7 peptides gave a clear response (figure 5.5).
Figure 5.4

Proliferation of pooled lymph node cells taken at day 7 from mice primed with pooled HPV16 L1 peptides (each pool consisted of the first four of the peptides shown on each figure) in complete Freund's adjuvant in the base of tail. Medium was IMDM with 10% FCS. Results are the mean of triplicates. Peptides were used at 120, 60, 30, 15 and 7.5 μg/ml, the highest concentration on the left.

Proliferation stimulated by L1 86-110 and by L1 305-329 is clearly statistically significant at all concentrations by calculation of standard error of the difference between mean control wells and mean experimental wells (p<0.001).
Figure 5.5

Proliferation of pooled lymph node cells taken at day 7 from mice primed with pooled HPV16 E7 peptides (each pool consisted of the first four of the peptides shown on each figure) in complete Freund's adjuvant in the base of tail. Medium was IMDM with 10% FCS. Results are the mean of triplicates. Peptides were used at 100, 20, 4 and 0.8 μg/ml, the highest concentration on the left. The fifth bar represents the control with no antigen. L1 indicates L1 peptide amino acids 60-84.

No statistically significant difference was found between proliferation with no antigen and proliferation with any concentration of the peptides. Although E7 78-98 appears to stimulate proliferation at the highest concentration, the standard error of difference of the means between the mean of 6 control wells and 3 peptide wells is 718 (p<0.1).
DNA vaccination

Assembly of constructs

Two constructs were made. One, pCDNA3.16L1, expressed HPV16 L1 and the other, pCDNA3.16E7, expressed HPV16 E7. HPV16 L1 DNA was removed as a fragment from pMAL-c16L1 (a gift from Geoff Higgins), the ends were blunted and it was inserted into pcDNA3. pCDNA3.16E7 was constructed by P. O'Brien (ICRF). Confirmation that the constructs were correct was obtained by DNA sequencing at the ends of the insert.

Vaccination

Mice were injected intramuscularly in both hind legs with 50μl of water containing 50μg of DNA. This was repeated at least once with intervals of four weeks and draining lymph nodes were obtained a further 10 days later.

Results of DNA vaccination

The para-aortic nodes were nearly always enlarged and the inguinal nodes were sometimes enlarged. About 10^7 lymph node cells were obtained from each mouse. Experiments were performed on groups of three or four mice. Groups were primed with both constructs on the same days so that they could act as controls for each other. In initial experiments lymph node cells from DNA-primed mice were cultured with peptides over a range of concentrations or with live vaccinia constructs over a range of concentrations as in experiments with human PBMC (chapter 3). In these experiments cells consistently responded to mitogen but not to the antigens.

Variation of medium, length of culture and length of time from final priming to harvesting of lymph nodes failed to yield any response in vitro. The experiments were repeated using CBA mice also with no responses.
Later a small amount of L1 VLP were obtained (from Mike Plumstead and Margaret Stanley) and L1 DNA vaccinated C57BL6 mice gave a clear response to this (figure 5.6).

![Figure 5.6](image)

**Figure 5.6**

Proliferation of pooled lymph node cells from mice immunised with pCDNA3.16L1 as described in the text. The approximate concentration of L1 VLP is given in µg/ml. The standard error of the difference between the mean of 12 wells with no antigen and that of 3 wells with 0.2µg/ml is 150 therefore the two means are significantly different (p<0.001).

This experiment could only be performed once because of time constraints and the availability of L1 VLP. The concentration of L1 VLP was approximated and it seems likely that a lower concentration would have produced much more striking proliferation. Insufficient primed cells were available to test this.
Discussion

It proved very difficult to establish a system of priming CD4 T cell responses to HPV16 antigens in mice. Even when a method was established using DNA immunisation these cells did not proliferate in response to synthetic peptides. The only lymphocyte proliferation stimulated by any of these peptides in vitro was of cells from peptide primed mice. Such responses may have no relevance to those produced by natural processing of the whole protein. Given that the methods used could reliably generate ovalbumin stimulated proliferation of ovalbumin primed mice, the reason why HPV16 protein immunisation was unsuccessful are unclear. The problem may lie with the method itself which, while fairly reliable with well documented complex antigens may be too insensitive to detect responses to less immunogenic proteins.

The method of priming mice by immunisation at the base of the tail with antigen in complete Freund's adjuvant and assessing proliferation of cells from draining lymph nodes was first described by Corradin et al. In a systematic evaluation the optimum conditions were established using tuna cytochrome c and human gamma globulin as antigens (Corradin, Etlinger, and Chiller 1977). However examination of recently published work using the method shows that while the vaccination protocol is almost always constant methods vary enormously in vitro. For instance as well as differences in cells and media different laboratories have pulsed with [3H]TdR as early as 48 hours (Rahemtulla et al. 1994), and as late as 120 hours (Grewal et al. 1997). In the original description it was found that experiments in round wells peaked at about 72 - 96 hours whereas if flat wells were used the peak was at 120 hours (Corradin, Etlinger, and Chiller 1977). Presumably optimum conditions need to be established for each experimental system used.

The chief problem, as with the human work, was the ineffectiveness of the chosen peptides in stimulating responses. For L1 protein this could well be because the
majority of the protein was not represented in the overlapping 25mer peptides (figure 5.2). This cannot be the reason for the failure of the E7 peptides to stimulate DNA-primed cells. When obtaining the sets of peptides the assumption was made that any peptide which includes the minimal epitope would be able to restimulate cells primed with the whole antigen. The set of E7 peptides was designed to include all possible sequences of 15 amino acids represented in the E7 protein and so, almost certainly, all possible class II restricted epitopes (figure 5.1). Evidence discussed in chapter 4 would suggest that this assumption was invalid for peptide stimulation of PBMC. One feature of the *in vitro* part of the Corradin method when used with peptides is that the cells are bathed in peptide at a concentration far higher than could ever be achieved *in vivo* (Sercarz et al. 1993). In fact these peptides would only be generated intracellularly under physiological conditions. It has been shown that presentation of peptides of 24 or more residues does not necessarily require processing, they can be presented by fixed, irradiated cells (Bhardwaj et al. 1992). It seems that the E7 peptides could not be effectively presented with or without processing.

There is published evidence for proliferative responses to HPV16 E7 in mice (Chambers et al. 1994, Comerford et al. 1991, Shepherd et al. 1992, Tindle et al. 1991) One study has addressed murine proliferative responses to HPV16 L1 (Davies et al. 1990) and one study to HPV16 E6 (Gao et al. 1994).

Tindle *et al* screened a series of overlapping 15-20mer peptides covering the whole predicted sequence of HPV16 E7 (Tindle et al. 1991). All peptides containing the sequence DRAHYNI (amino acids 48-54) stimulated proliferation of lymph node cells from mice primed with the peptide of amino acids 44-62. This peptide could both prime mice to respond to a MS2 HPV16 E7 fusion protein and could stimulate cells primed with this protein. Most of this work was done with B10.A(2R) mice which express I-A^k-I-E^d. Peptide responses to peptide primed mice were confirmed in a wide variety of genetic backgrounds.
Comerford et al used slightly different methods and a different set of overlapping 20mer peptides (Comerford et al. 1991). After priming CBA (I-A^K-E^K) mice with GST E7 fusion protein (obtained as insoluble bacterial inclusion bodies) or protein A E7 fusion protein, responses were found from several peptides but, in agreement with the report of Tindle, the greatest responses came from peptides which included the DRAHYNI motif.

The paper by Shepherd et al highlights problems with these methods (Shepherd et al. 1992). A series of experiments were conducted priming different strains of mice with GST E7 fusion protein and reading out on the same peptides as those used by Comerford et al. The results when positive are of low amplitude and vary not only between mouse strains but also for the same strain in different experiments. In addition E7 protein when cleaved from GST and further purified did not prime mice for peptide responses.

Using a completely different experimental system Chambers et al primed BALB/c mice with a skin graft which expressed HPV16 E7. A DTH-type response was then induced in an ear 7 days later by intradermal injection of vaccinia E7 (Chambers et al. 1994). Cells from the lymph nodes draining this ear and the graft were found to proliferate strongly when cultured with yeast-derived HPV16 E7. The chief finding of this study was that if the number of cells in the initial graft was below a certain threshold there was no DTH response to the later challenge. This unresponsiveness was sustained for at least a month, even if a later graft was used with a higher cell number.

Gao et al primed C57BL and DBA/2 mice to HPV16 E6 by scarification with recombinant vaccinia at the base of the tail (Gao et al. 1994). Lymph node cells were obtained from the draining nodes and stimulated with each of a set of overlapping 10 mer peptides covering the whole of E6. No positive responses were found with DBA (H2^d) mice. Two of the 30 peptides caused proliferation of about twice the
background when C57BL (H2b) mice were used. These peptides are much shorter than many known class II restricted epitopes.

Davies et al immunised CBA, BALB/c and (CBA × BALB/c) F1 mice with the set of L1 peptides described by Strang et al containing putative class II binding motifs (Davies et al. 1990, Strang et al. 1990). Three of these eight peptides could stimulate proliferation of peptide primed lymphocytes *in vitro* in all three mouse strains. Two of these three peptides are represented in the set of L1 peptides tested in the experiments above. One gave moderate responses and one gave no responses, the other positive responding peptide described above was not represented in Davies' set.

In some of the studies of HPV16-specific proliferation described above considerable variation was reported from experiment to experiment when apparently identical conditions were used for each (Davies et al. 1990, Shepherd et al. 1992). A clue which may help to explain these variations has come in a recent publication describing marked differences in results of proliferation assays which are apparently caused by housing mice singly or in groups (Grewal et al. 1997). In this study a well established and reproducible system was used: mice were primed with hen egg lysozyme using the method of Corradin and proliferation was measured to peptides known to be class II restricted epitopes. Although this observation applied to male mice only it demonstrates that the system is complex and poorly understood.

Intramuscular injection of plasmid DNA encoding antigens can stimulate long-lasting humoral and cellular immunity (Ulmer 1993). Recently it has been shown that immunisation of rabbits with plasmid DNA encoding CRPV L1 can protect recipients from infectious challenge with the virus (Donnelly et al. 1996). DNA vaccination seems an ideal way to bypass difficulties in producing protein antigen. pCDNA3 is a plasmid which has proved to be successful for DNA vaccination (Anderson et al. 1996). It has a CMV promoter and should allow high level expression in murine cells.
Recent observations of DNA vaccination have suggested that the immunogenicity of plasmid DNA depends on the presence of certain immunostimulatory DNA sequences (Sato et al. 1996). It has been demonstrated that unmethylated CpG dinucleotides can have an adjuvant-like effect in directing responding CD4 T cells to produce IFN-γ (Yi et al. 1996). The plasmid used in these experiments - pcDNA3 - does contain sequences of this type. These observations are quite new and it is likely that more subtleties controlling the immune response to DNA vaccination remain to be discovered.

In conclusion, although a system to study HPV16 responses in DQ transgenic mice was not established, attempts to do this have resulted in useful methodological observations. Firstly it has been shown that polynucleotide immunisation in the hind legs of mice generates a response in the para aortic lymph nodes and that cells taken from these nodes will proliferate in vitro when cultured with the protein antigen encoded within the DNA used for priming. This result has not been described before and will be useful in investigating DNA immunisation generally.

Secondly, the difficulties encountered could be because of the unreliability of the available methods for studying primed CD4 T cell responses in mice. Mice may not have been primed by the protein antigens or the peptides used may have been unable to restimulate primed T cells in vitro. It is suggested that there are limitations on the use of synthetic peptides to detect and map class II restricted responses and that these are caused by the unpredictable effects of peptide residues outside the relevant minimum epitope.
Chapter 6

Discussion
Discussion

The results presented in this thesis provide support for the idea that CD4 T cell responses primed by natural infection with HPV16 can sometimes be detected in the peripheral blood but that they are transient. It is not possible to say whether they perform an important functional role and nothing is known of their HLA restriction or cytokine production.

An effective vaccine to prevent or attenuate HPV16 infection must be able to prime CD4 T cells in such a way as to direct the right sort of immune response. Given our present lack of knowledge about natural immune responses to HPV16 and in particular about the nature and effectiveness of HPV16-specific CD4 T cells it is still not clear how this should be achieved or even, perhaps, what the right sort of immune response is.

It remains uncertain which immune effector cells control or eradicate HPV. In BPV, CRPV and COPV there is evidence that protection, at least in the short term, is accomplished by IgG antibody. It may be that this is produced by B cells using Th1-type CD4 T cell help although for humans there is no good evidence that such antibodies require IFNγ or other Th1-type cytokines (Abbas, Murphy, and Sher 1996) (in mice it seems that they do as the IgG isotypes produced in response to viral infections are different in IFNγ-deficient or IFNγ receptor-deficient mice (Graham et al. 1993, Huang et al. 1993, Kundig et al. 1993)). Even in BPV4 and CRPV where early regression of papillomas has been induced by immunisation with E7 or with E1 and E2 it is not clear exactly how this was mediated. Characterisation of the infiltrating cells in a regressing lesion can suggest which effectors are acting (for all papillomaviruses studied the suggestion is CD4 T cells) but it is possible that a minority population of cells is more important.
The original objective of this study was to detect and characterise CD4 T cells which had been primed by natural infection with HPV16. The questions this raises remain important and have been addressed by several groups around the world but they have not been answered. So why is it so difficult to detect these cells? It is possible that they never enter the circulation. HPV16 causes a very localised infection and there is no need for a systemic response. The findings described here suggest that if primed cells do enter the circulation they may only be present transiently. The mechanism for maintaining T cell memory remains controversial (Zinkernagel et al. 1996), it is possible that these primed cells are not restimulated and so do not persist for long anywhere in the body.

If there is only a small window of time for detection perhaps most work has been carried out on the wrong group of subjects. The findings of this thesis would suggest that, at least for L1-primed cells, rather than concentrating on women with HPV disease (usually manifest as CIN or cervical carcinoma) we should look at subjects who are much younger, those who the recent cohort studies suggest have the highest incidence of primary infection (Schiffman et al. 1996).

If circulating CD4 cells primed to respond to HPV16 are only present transiently in the peripheral blood it would explain some of the anomalies in the published literature. For example the report of Altmann et al. (Altmann et al. 1992) contains the only convincing published description of PBMC proliferation to HPV16 antigens as bacterial fusion proteins in a conventional 5 day assay. Several other groups have reported their inability to achieve this and others have tried but not reported their negative results. The logical and soundest way for Altmann et al. to continue would have been to restimulate these proliferating cells, however for the rest of the reported work they resorted to using T cell lines generated with pooled peptides which, for the reasons discussed in chapter 4, is much less satisfactory. It is very likely that the initial observation was carried out around the peak of a transient response and that it could not be repeated at other times or in other individuals.
In chapter 3 it was shown that whereas all donors tested made some response to whole inactivated influenza virus only three of five donors responded to recombinant vaccinia containing influenza nucleoprotein (VVNP). The most likely explanation for this is that the immunodominant response in the two non-responders was to another part of the virus. This illustrates another possible reason for difficulty in detecting HPV16 responses. There are no reported studies which have looked for CD4 T cell responses to any HPV16 antigens other than E6, E7 or L1. This leaves a large part of the virus untested. The clearest report of CD4 T cell responses to HPV is described for HPV1 where large quantities of whole viral antigen can be obtained (Steele, Stankovic, and Gallimore 1993). In this study CD4 T cell clones were established which were specific for HPV1 E4. In rabbits it has been shown that immunisation with E1 and E2 proteins can trigger tumour regression (Selvakumar et al. 1995).

A further possibility is touched on in the unexplained findings described in chapter 4 that prolonged incubation of PBMC with recombinant vaccinia expressing E6 and E7 results in inhibition of [\(^3\)H]TdR incorporation without cell death (as determined by trypan blue exclusion). This is only partly explained by mycoplasma contamination. It is possible that as part of a strategy of immune evasion these HPV16 proteins can in some way inhibit an immune response. However, there is no convincing evidence for this and, as stressed in the discussion of chapter 4, the vaccinia assay uses a very complex and poorly characterised system. It is possible that these findings have a trivial explanation.

There seems to be a difference between immune responses to HPV16 and immune responses to other papillomaviruses at least in our ability to detect and investigate them after natural infection. It is generally accepted that the reason for the relative oncogenicity of HPV16 and the other "high risk" viruses compared with the "low risk" viruses is the well documented superior transforming ability of their E6 and E7 proteins (Storey et al. 1988). If "high risk" viruses were better able to persist because
of an enhanced ability to evade immune responses (at least in some individuals) this could also explain their relative oncogenicity.

There is no clear evidence that HPV16 proteins are intrinsically less immunogenic than other proteins of similar size and composition. HPV16 E7 has successfully been used as a tumour rejection antigen in animal models (Chen et al. 1991, Meneguzzi et al. 1991). However papillomaviruses have a strict tissue and species tropism and the fact that the proteins are antigenic in rodents does not necessarily imply that they will be in humans. As has been stressed, HPV hs evolved together with the human immune system and some or all HPV proteins could have evolved sequences which are minimally immunogenic across a range of HLA backgrounds. It is reassuring that HPV16 proteins do appear to be immunogenic in some individuals when given in preliminary human vaccine trials (Borysiewicz et al. 1996) Tindle

**Viral persistence indicates a high risk of CIN**

Infection with oncogenic HPV is now known to be very common indeed, more than 20% of women are infected at some time and perhaps as many as 50% (Schiffman et al. 1996). Why then does it only lead to cervical carcinoma in a small proportion of cases? The key seems to be persistence of the virus either because it is not eradicated or because it has already set in train the early stages of carcinogenesis.

Not uncommonly individuals are found who have evidence of pre-malignant change in more than one anogenital site. The simple idea that this is caused by a particularly virulent strain of HPV can probably be discounted as this would lead to geographical clustering of the phenomenon. The explanation must be that these individuals have a predisposition to HPV-induced oncogenesis. This could be due to a cellular defect which allows HPV quickly to initiate transformation. Perhaps more likely it is caused by a failure of immune-mediated control of infection leading to viral persistence.
Latency

Resolution of a visible papilloma does not necessarily correlate with elimination of the virus from the site and several lines of evidence suggest that HPV latency occurs.

1/ Warts are commonly seen to appear almost simultaneously at multiple sites, this is unlikely to be caused by multiple simultaneous infections.

2/ Viral DNA has been detected at morphologically normal sites (Ferenczy et al. 1985).

3/ Immunosuppression has been shown to be associated with extensive warts suggesting that there may normally be a latent state controlled by cellular immunity (Frazer and Tindle 1992).

4/ BPV1 forms multicopy plasmids in mouse fibroblasts which it has transformed. These plasmids replicate only once per cell cycle, apparently under viral control. Further investigation has confirmed that BPV1 does suppress its own replication (Nallaseth and DePamphilis 1994). If this control were manifest in vivo it would be a very effective way of maintaining latency.

5/ There is evidence that HPV16 is often transmitted vertically at birth and that in these infants it remains detectable for months or years (Cason et al. 1995, Puranen et al. 1996) although this has been disputed (Wideroff et al. 1996). If there is persistence of virus in such infants it implies latent infection.

Latency maintained at very low copy number would be very difficult or impossible to detect even by in situ hybridisation and its role in the natural history of infection is hard to investigate since detection would require excision biopsy. If latency is indeed part of the natural history of papillomavirus infection it has important implications for the target of intervention to control oncogenic HPV infection. It is
the difference between the attempted maintenance of "sterile" immunity with circulating antibody as the chief effector and the attempted control of a latent intracellular infection perhaps by specific cellular immunity.

The demonstration of vertical transmission also suggests the possibility that exposure of infants to HPV16 antigens at birth leads to a degree of tolerance, or to a greater susceptibility to HPV16 in later life. This possibility would be very difficult to examine epidemiologically because of the long time scale involved. It could explain the paradox that an almost ubiquitous infection causes cervical carcinoma only in an apparently arbitrary minority of women.

**Immunotherapy and prevention**

Intervention in HPV disease could be by prophylactic immunisation with the intention of preventing HPV infection - an approach which seems to have been successful in the short term in animal models. Or by immunotherapy - stimulating the immune system to alter the course of established infection. Trials of both these forms of intervention have started in recent years.

**Prophylactic immunisation**

As described in the introduction immunisation with the capsid proteins is able to protect the subject from infectious challenge with species specific papillomavirus infection in cattle, rabbits and dogs. In many human subjects new detection of HPV16 on the cervix is followed after a delay by the detection of HPV16 L1 antibody in the blood(Wikstrom et al. 1995). Preliminary clinical trials of HPV16 VLP are starting or are about to start in USA and UK.

Ideally prophylactic vaccination should be carried out on a population which has not been exposed to the virus and if vaccination proves effective the aim would probably be to target children. For reasons already discussed it is difficult or impossible to find
any group, including infants, which has definitely not been exposed to oncogenic HPV.

If prophylactic vaccination proves ineffective, perhaps because of widespread unrecognised latent infection, an alternative approach is to attempt to control established infection or established tumour by immunotherapy.

Immunotheapy

Intervention by vaccination to alter the progress of established tumours or infectious disease is a clinical goal of much immunological research. To date, in spite of the promise of animal experiments there has been very little success in humans. The E6 and E7 proteins are appealing targets for immunotherapy of HPV associated tumours because tumour growth requires their continuous expression and they have been shown to be tumour rejection antigens in rodent models (Chen et al. 1991, Meneguzzi et al. 1991). Immunisation with early gene products has been shown to accelerate regression of papillomas caused by BPV4 or CRPV. One problem for human immunisation is that the E6 and E7 proteins are potentially tumorigenic and so may not be safe to inject in an unaltered form.

Ultimately the only way to know whether this kind of approach can be used to treat established human tumours is to try it and recently several such trials have started. A phase1/11 trial of recombinant vaccinia expressing HPV16 E6 and E7 (in an attenuated form) has shown that this vaccine was harmless in the patients tested, that it did not escape into the environment and that in one patient at least it was immunogenic (CTL to HPV18 were generated) (Borysiewicz et al. 1996). A trial is underway on cervical cancer patients in Holland using two peptides derived from HPV16 E7 which have been shown to generate HLA*0201 restricted CTL in vitro and in HLA*0201 transgenic mice (Ressing et al. 1995). Further results using these and other approaches are awaited with interest. If a trial like this is able to generate
HPV16-specific immunity it will provide a short cut towards investigating the CD4 T cell response to the virus.

Considerable difficulties have been encountered in seeking to find and to characterise CD4 T cell responses to HPV16 and possible reasons for this have been discussed. The findings of this thesis suggest some ways in which these questions could be addressed in the future. The recombinant vaccinia method could easily be used to screen a large number of younger subjects to look for transient L1-specific responses at the time of primary infection. If a response is detected at its height it may be possible to restimulate the responding cells and to generate T cell clones to allow further analysis. The vaccinia method could also be valuable in the investigation of subjects vaccinated in a preliminary VLP vaccine trial. Finally, the demonstration in chapter 5 of the power of DNA vaccination to generate immune responses supports the idea that vaccination with DNA encoding multiple epitopes may prove to be the most effective clinical measure against HPV16.
References


Ellis, J.R.M., P.J. Keating, J. Baird, E.F. Hounsell, D.V. Renouf, M. Rowe, D. Hopkins, M.F.


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Appendix

The appendix contains details of all the results of proliferation of whole PBMC of all of the laboratory donors and HPV16-positive patients using the vaccinia proliferation assay described in the introductions to chapters 3 and 4. These results are summarised in figures 4.2 and 4.5.

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Donor LA

**Graph:**
- **No antigen**
- **VVWT**
- **VVCd1**
- **VVE7**

**Y-axis:** Counts per minute

**X-axis:** MOI (multiplicity of infection)

Values:
- 0.001, 0.01, 0.1, 1
Donor LC

Counts per minute

- ■ — No antigen
- ▲ — VVWT
- × — VVCd1
- ● — VVE7

MOI
Domor LE (22 months before figure 4.4)

Counts per minute

- No antigen
- VVWT
- VVCd1
- VVE7

MOI
Donor LE (17 Months before figure 4.4)

- No antigen
- VVWT
- VVCd1
- VVE7
- VVE6
- VVL1

Counts per minute

MOI
Donor LE (Time 0 in figure 4.4)

- No antigen
- VVWT
- VVCd1
- VVE6
- VVE7
- VVL1
- VVNP

Counts per minute

MOI
Donor LE (Time 1 week in figure 4.4)

- ■ No antigen
- ▲ - VVWT
- ● - VVCd1
- × - VVE6
- VVE7
- VVL1
- VVNP
Donor LE (Time 5 weeks in figure 4.4)

- No Antigen
- VVWT
- VVCd1
- VVE6
- VVE7
- VVL1
- VVN1
Donor LG

Counts per minute

- No antigen
- VVWT
- VVCD1
- VVE6
- VVE7
- VVL1

MOI

0.001 0.01 0.1 1
Donor LH

Counts per minute

MOI

- No antigen
- VVWT
- VVCd1
- VVE6
- VVE7
- VVL1

0 1

0.1 1

0 500 1000 1500 2000 2500 3000
Donor LL

Counts per minute

MOI

- ■ — No antigen
- ▲ - VVWT
- × - VVCd1
- * - VVE6
- ○ - VVE7
- • - VVL1
Donor MK

![Graph showing counts per minute vs. MOI for different antigen treatments.](image)

- No antigen
- VVWT
- VVCD1
- VVE6
- VVE7
- VVL1

Counts per minute

MOI
Donor WD

Counts per minute

No antigen
VVWT
VVCd1
VVE6
VVE7
VVL1

MOI

0.001 0.01 0.1 1 10

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Donor WE

- NO ANTIGEN
- VVWT
- VVCd1
- VVE6
- VVE7
- VVL1

Counts per minute

MOI