



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Construction and characterisation of a recombinant vaccinia virus expressing human papillomavirus proteins for immunotherapy of cervical cancer

M.E.G. Boursnell*, E. Rutherford*, J.K. Hickling*, E.A. Rollinson*, A.J. Munro*, N. Rolley*, C.S. McLean*, L.K. Borysiewicz†, K. Vousden‡ and S.C. Inglis*§

The presence and consistent expression of the genes encoding the human papillomavirus (HPV) E6 and E7 proteins in the great majority of cervical tumours presents the opportunity for an immunotherapeutic approach for control of the disease. This report describes the construction and characterisation of a recombinant vaccinia virus designed to express modified forms of the E6 and E7 proteins from HPV16 and HPV18, the viruses most commonly associated with cervical cancer. The recombinant virus (designated TA-HPV) was based on the Wyeth vaccine strain of vaccinia, and was shown to express the desired gene products. Studies in mice indicated that the recombinant virus was less neurovirulent than the parental virus and was capable of inducing an HPV-specific CTL response. This pre-clinical evaluation has provided a basis for the initiation of human trials in cervical cancer patients. Copyright © 1996 Elsevier Science Ltd.

Keywords: HPV; vaccinia virus; cervical carcinoma

Over the past decade it has become increasingly clear that cervical cancer is closely linked to infection by certain types of human papillomavirus (HPV)¹. This conclusion is based on several lines of evidence, including the observation that over 90% of cervical tumours contain HPV DNA. Over 60 different types of HPV have now been described, but only a limited number are associated with tumours. Of these “oncogenic” HPVs, the most commonly found are HPV16, which occurs in some 50–60% of tumours, and HPV18 which occurs in about 20–25% of cases². A number of other HPVs are also found, albeit more rarely, and these include HPVs 31, 33, 35, 45, 52, 39. Infection with these “oncogenic” viruses however, does not necessarily lead to tumour development. A substantial proportion of healthy women show evidence of infection, and the vast majority of these infections do not lead to malignant disease. It appears rather that the presence of these viruses in the genital tract may, over a long period of time predispose to the development of cervical cancer by providing one of the elements in a multi-stage process of tumourigenesis.

*Cantab Pharmaceuticals Research Ltd., Cambridge Science Park, Cambridge CB4 4GN, U.K. †Department of Medicine, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, U.K. ‡National Cancer Institute, Building 539, West 7th Street, Frederick, MD 21702-1201, U.S.A. §To whom correspondence should be addressed.

Cervical tumour cells generally do not contain the complete HPV genome, but a consistent feature is the retention and expression of the portion of the virus genome encoding two of the early virus proteins, E6 and E7³. An increasing body of evidence indicates that the expression of these proteins contributes to the transformation process. *In vitro* studies have shown that the E6 and E7 genes from the oncogenic HPVs can immortalise cells in culture^{4,5}, and both proteins are known to interact with and inhibit the activity of cellular proteins that control cell division; E7 binds to the cellular retinoblastoma (Rb) protein^{6,7} and E6 binds to cellular p53⁸. Furthermore recent studies have suggested that continued expression of E6 and E7 within tumour cells is required to maintain the transformed state⁹.

Regardless of their role in transformation however, the presence of the E6 and E7 proteins in cervical tumour cells offers an unusually clear cut opportunity for cancer immunotherapy, since they represent genuine tumour-specific antigens that could act as targets for destruction of tumour cells without damage to healthy host cells. Since both the E6 and E7 proteins are intracellular, the immune effector mechanism which is most likely to be effective against cervical tumour cells is the cytotoxic T lymphocyte (CTL). CTLs can destroy cells expressing foreign antigens through recognition of peptide fragments generated within the cell and transported to the cell surface and presented by major histocompatibility complex (MHC) class I antigens¹⁰.

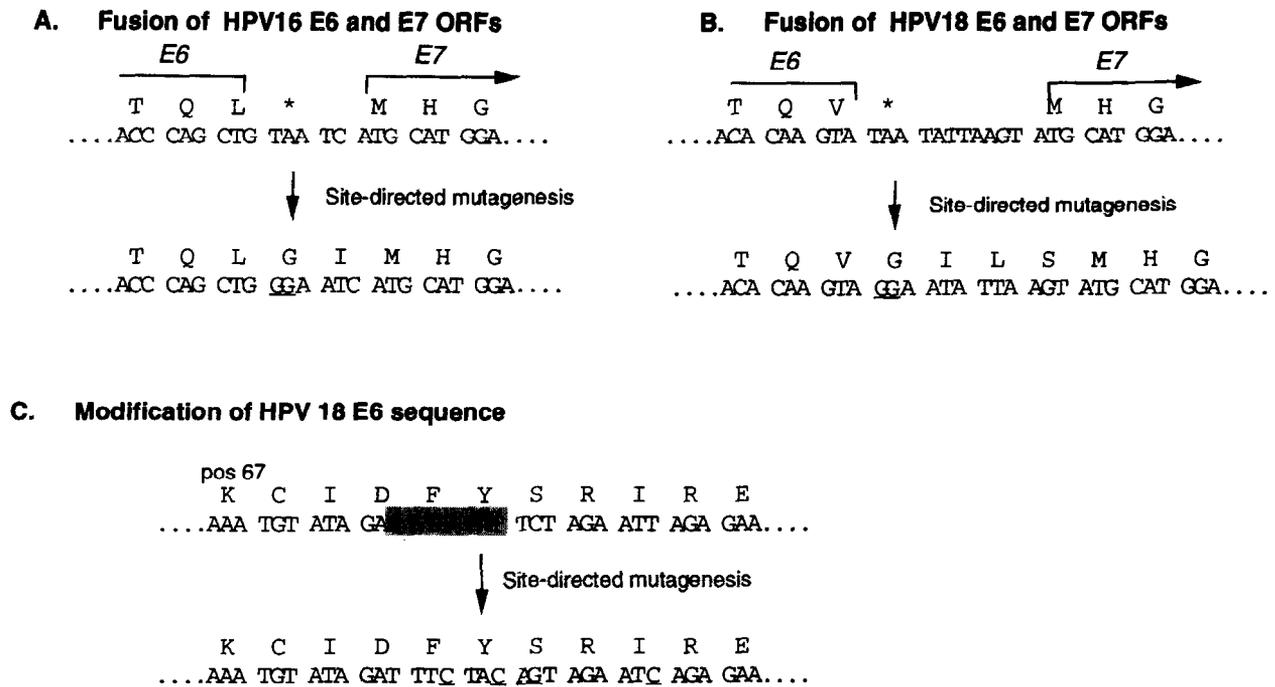


Figure 1 Fusion of the E6 and E7 genes. (A) HPV16; (B) HPV18 The nucleotide changes (underlined) were confirmed by nucleotide sequencing; (C) Modification of the HPV18 E6 coding sequence to eliminate the potential transcription termination sequence TTTTAT (shaded) and to reduce direct homology with the corresponding HPV16 sequence. The nucleotide changes shown (underlined) were confirmed by nucleotide sequencing.

A number of studies have indicated that CTL are potentially very powerful agents of tumour cell destruction^{11,12}. Furthermore, experimental data from animal models have indicated that the HPV E6 and E7 proteins can act as targets for CTL-mediated tumour cell killing¹³⁻¹⁶.

Induction of an antigen-specific CTL response generally requires that the target antigen is produced inside host cells, in order that proper antigen processing and MHC class I-mediated presentation can occur. This can be accomplished through the use of a recombinant virus vector which carries the gene for the target antigen.

In this report we describe the design, construction and characterisation of a recombinant vaccinia virus expressing the E6 and E7 proteins from HPV types 16 and 18 specifically for the purpose of inducing a beneficial immune response in humans. The results of early stage clinical studies with this virus are presented in a separate report (Borysiewicz *et al.*, submitted for publication).

MATERIALS AND METHODS

Virus and cells

The parent virus used for construction of TA-HPV was the Wyeth (New York Board of Health) strain of vaccinia virus, obtained from the American Type Culture Collection (ATCC), ref: VR325. Vero cells were obtained from the European Collection of Animal Cell Cultures (ref: 84113001). MRC-5 cells were obtained from the National Institute of Biological Standards (NIBSC), UK.

Cloning and mutagenesis of the HPV16 and HPV18 E6 and E7 genes

The E6 and E7 genes of HPV lie adjacent to one another on the virus genome. A DNA sequence

including the complete coding sequences for E6 and E7 was cloned from the HPV16 genome by polymerase chain reaction (PCR) into the plasmid vector pIMS5 to generate pIMS7. To facilitate cloning, the primers for the PCR were chosen to introduce an NcoI restriction site around the start codon (position 83) of the HPV16 E6 gene¹⁷ (CC ATG G), and a SmaI site immediately downstream of E7. An equivalent strategy was used to introduce a restriction site around the start codon (position 105) of HPV18 E6¹⁸, and to clone the HPV18 E6 and E7 coding sequences, yielding the plasmid pIMS8.

The plasmids were modified by standard site directed mutagenesis methods¹⁹; a) to create fused E6 and E7 coding sequences for both HPV16 and HPV18; b) to introduce mutations at the Rb binding site of E7 and c) to eliminate a potential termination signal for early vaccinia virus transcription in the HPV18 E6 coding sequence (Figure 1, Figure 2). These manipulations generated the plasmids pIMS7.1 (fused, unmodified HPV16 E6/E7 coding sequence), pIMS7.2 (fused, modified HPV16 E6/E7 coding sequence), pIMS8.1 (fused, unmodified HPV18 E6/E7 coding sequence) and pIMS8.2 (fused, modified HPV18 E6/E7 coding sequence).

Analysis of Rb protein binding

Plasmids pIMS7.1, pIMS7.2, pIMS8.1 and pIMS8.2 were modified by site directed mutagenesis to remove the E6 coding sequences, and to place the E7 gene adjacent to the phage T7 RNA polymerase promoter, generating plasmids pIMER10, pIMER11, pIMER12, pIMER13 respectively. The plasmids were added to a coupled *in vitro* transcription/translation system (TNT, Amersham International), and the protein products tested for Rb

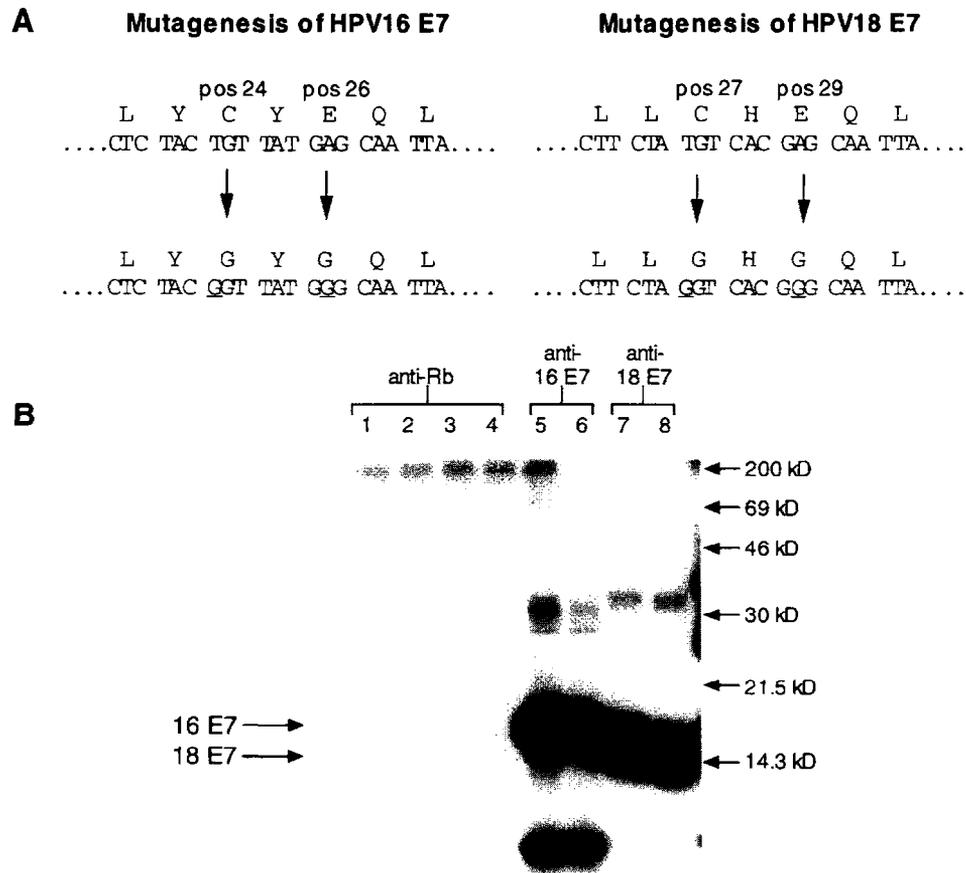


Figure 2 A. Modification of the Rb binding site of the HPV16 and HPV18 E7 genes. The nucleotide changes shown (underlined) were made by oligonucleotide-mediated site-directed mutagenesis and confirmed by nucleotide sequencing. B. Modified or unmodified forms of HPV16 E7 and HPV18 E7 were produced and 35S-met labelled in a rabbit reticulolysate system. The products of the reaction were immunoprecipitated with either anti-Rb, anti-16 E7, anti-18 E7. The original reactions contained radiolabelled; HPV16 E7 (lanes 1 and 5); modified HPV16 E7 (lanes 2 and 6), HPV18 E7 (lanes 3 and 7), modified HPV18 E7 (lanes 4 and 8).

binding²⁰. In brief, unlabelled Rb protein was produced by *in vitro* translation of the plasmid pcRb²⁰ and mixed with radiolabelled *in vitro* translated HPV E7 protein. The mixtures were then immunoprecipitated using antibodies directed against Rb (monoclonal antibody IF8—a gift from Professor David Lane, Dundee University), or against HPV 16 E7 (polyclonal anti-HPV16 E7—a gift from Dr J. Morris, Ludwig Institute for Cancer Research, London), or against HPV 18 E7 (cocktail of monoclonal antibodies 17A6, 7E10, 17C4, 10A8, from Dr R. Tindle, Lions Human Immunology Laboratories, Brisbane). Precipitates were analysed by SDS polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Assays of transformation activity

This was assessed using an assay based on co-transformation of rat embryo fibroblasts with activated ras oncogene²¹. Coding sequences corresponding to the fused HPV16 E6/E7 genes with and without Rb binding site mutations were excised from the plasmids pIMS7.1 and pIMS7.2 respectively using HindIII and BamHI. These fragments were cloned into each of two mammalian cell expression vectors, pJ3 and pJ4 which allow expression of heterologous genes under the control of the SV40 promoter and the Moloney leukaemia LTR promoters respectively²². Control plasmids containing the wild type E7 coding sequences, pJ316E7 and pJ416E7, were described previously²³. Transformation

assays contained 5 μ g of the test plasmid, 5 μ g of an expression plasmid containing the Harvey ras oncogene (pEJ6.6,²⁴) and 1 μ g of the plasmid pSV2neo. Surviving colonies were counted after 2 weeks in culture.

Construction of TA-HPV

A plasmid pIMMB7a was constructed which contained a 1kb sequence spanning symmetrically the chosen insertion site within the vaccinia virus genome (Figure 3) (prepared directly from vaccinia virus DNA by PCR). pIMMB7a also contained the guanine phosphoribosyl transferase (gpt) gene under the control of the vaccinia virus 7.5K gene promoter²⁵, and the origin of replication for the phage f1. A SnaBI site was introduced at the chosen insertion site within the vaccinia specific sequence (pIMMB8a). The final recombination vector (pIMMB9a—Figure 4) for insertion of the E6/E7 coding sequences into the vaccinia genome was derived from pIMMB8a by insertion at the SnaBI site, of a DNA cassette with the following elements: the fused, modified HPV16 E6/E7 sequence (from pIMS7.2) under the control of the vaccinia specific H6 promoter²⁶, and, in a head-to-head orientation, the fused modified HPV18 E6/E7 sequence from pIMS8.2 under the control of the vaccinia specific 7.5K promoter.

A two-stage transient dominant selection procedure²⁷ was used to recombine the HPV genes into the vaccinia genome (Figure 4). Virus isolates generated by this procedure were screened for the presence of the desired

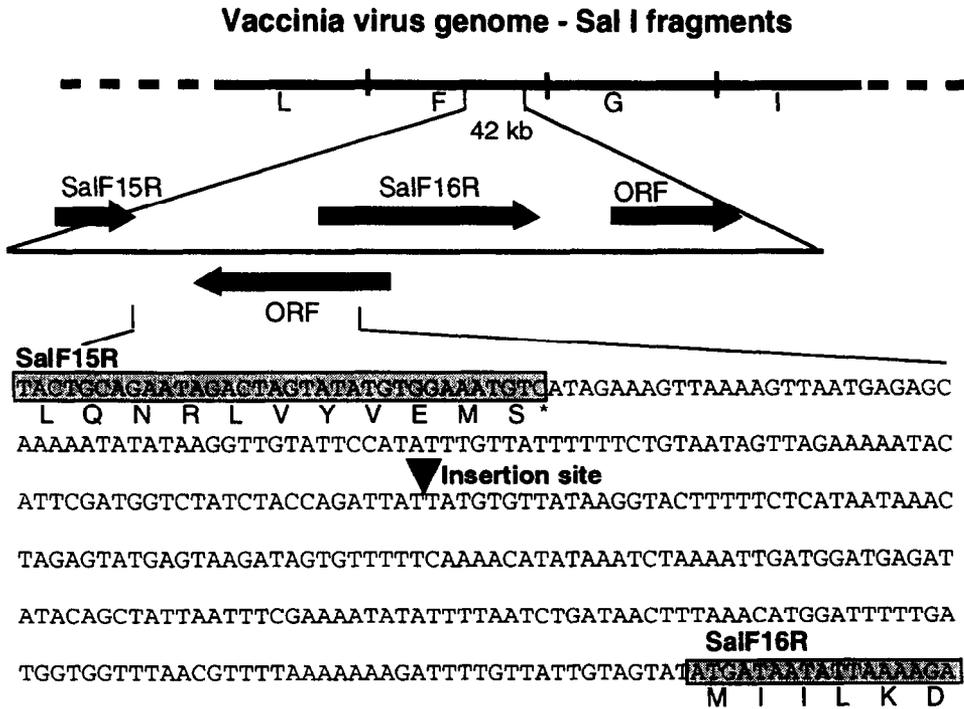


Figure 3 Location of the site within the SalIF fragment of the vaccinia virus genome selected for insertion of the HPV sequences. Open reading frames are indicated by hatched arrows and shaded boxes.

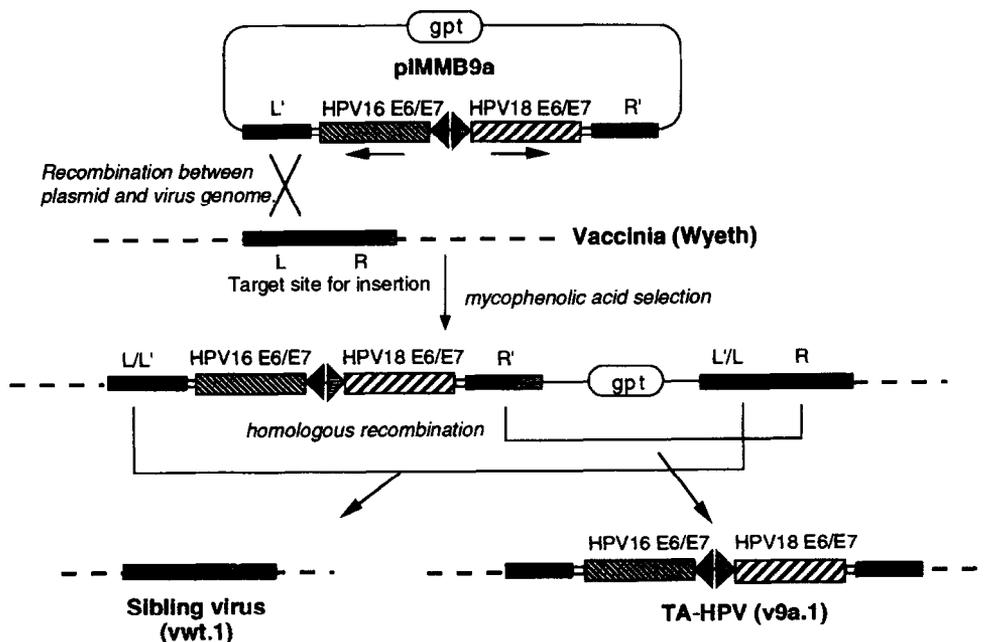


Figure 4 Introduction of HPV coding sequences into the vaccinia virus genome. Plasmid pIMMB9a was transfected into Vero cells, and the cells were co-infected with parental vaccinia virus. Progeny virus containing a complete copy of the plasmid was isolated by mycophenolic acid selection, and then passaged in the absence of selection, giving rise to the virus v9a.1 (TA-HPV) containing the HPV gene expression cassette, and to a "sibling" virus exactly equivalent to v9a.1 except that it contains no HPV insert (vwt.1).

HPV insert by PCR, and from this analysis a single recombinant virus, v9a.1, was selected for further characterisation.

Purification and analysis of vaccinia virus DNA

Vaccinia virus-infected Vero cells were harvested, resuspended in PBS and the virus released by 3 cycles of freeze-thawing. Nuclei were removed by centrifugation at 300g for 2.5 min, and virus was recovered from the resulting supernatant by centrifugation in a

microfuge for 15 min. The pellets were resuspended and incubated in buffer containing proteinase K (0.5mg/ml) before extraction with phenol/chloroform/isoamyl alcohol. The DNA was recovered by ethanol precipitation.

Initial genomic characterisation was carried out by PCR analysis. Subsequently the nucleotide sequence of the insert was determined by cycle sequencing using a commercially available kit (Promega Inc.) and a set of 12 oligonucleotide primers designed to cover the HPV coding sequences and their respective promoters.

Western blotting

MRC-5 cells (5×10^6) were infected with v9a.1 or Wyeth strain vaccinia virus at a multiplicity of 10 pfu/cell, and incubated for 24h. The infected cells were harvested, recovered by centrifugation and resuspended in SDS-PAGE sample buffer. Lysates were subjected to PAGE and the gel contents transferred to nitrocellulose by electroblotting. The blot was washed in phosphate-buffered saline (PBS) containing 1% milk protein, rinsed in PBS, and incubated with the appropriate primary antibody diluted in PBS containing 0.5% Tween 20 (PBST) and 1% bovine serum albumin (BSA) for 1h at room temperature. HPV16 and HPV18 E7 sequences were detected respectively using the antibodies Camvir 3 (a gift from Dr M. Stanley, Department of Pathology University of Cambridge), and 7E10. An antibody against the HPV18 L1 protein (F1/1.1.16) was used as a negative control. The blot was further washed in PBST and incubated for 1h with goat anti-mouse horseradish peroxidase-conjugated antibody, in PBST containing 1% sheep serum. After final washing with PBST labelled bands were revealed by staining with diaminobenzidine tetrahydrochloride (DAB/H₂O₂). Radio-labelled protein molecular weight markers were detected by autoradiography of the nitrocellulose sheet.

Virulence studies in mice

Intranasal inoculation: Groups of 18 mice were infected intranasally with $10^{6.7}$ pfu of either TA-HPV or parental Wyeth virus. Six animals from each group were sacrificed on day 1, day 3 and day 14, and the lungs from each animal were removed for analysis. After storage at -70°C , the amount of virus present was assessed by standard plaque assay.

Intracranial inoculation: Groups of 12 animals were injected intra-cranially with three different doses of TA-HPV (10^5 , 10^6 , 10^7 pfu) or of the Wyeth parental virus. A further group of 12 animals was inoculated with PBS to serve as a negative control. Survival of the animals was monitored twice daily for 21 days.

Cytotoxic T cell induction

Female C57BL/6 mice (age 6–8 weeks) were immunised 3 times intra-peritoneally with 5×10^6 pfu of either TA-HPV, parental strain Wyeth vaccinia virus, or vE710, a recombinant vaccinia virus expressing the HPV16 E7 sequence²⁸ (a gift from Dr S.N. Stacey, Paterson Institute for Cancer Research, Manchester, UK). The only modification to the E7 sequence in vE710 from the wild type was at the N terminus, (Met-Gly-Ser-Pro-Gly, in place of the wild type Met-His-Gly). PBS immunised mice were also used as a control. Nine days after the final immunisations, the mice were killed, and single cell suspensions prepared from the spleens. Cells were cultured ($2.5 \times 10^6/\text{ml}$) in the presence of $10 \mu\text{M}$ synthetic peptide corresponding to residues 49–57 of HPV16 E7 (E7.49–57: R A H Y N I V T F), which represents an epitope recognised by HPV16 E7 specific H-2^b restricted CTL^{13,29}. Cultures were restimulated weekly by harvesting and re-culturing the cells at $6 \times 10^5/\text{well}$, in the presence of a 3×10^6 syngeneic spleen cells (mitomycin C treated), $5 \mu\text{M}$ peptide and supernatant from con A activated rat splenocytes as a source of IL2 (10IU/ml; Sigma). All cultures were maintained

in RPMI 1640 supplemented with $100 \mu\text{M}$ penicillin / streptomycin, 2 mM L-glutamine, $50 \mu\text{M}$ 2 mercaptoethanol and 10% heat inactivated foetal calf serum.

Chromium release assays

Target cells were either RMA (H-2^b Rauscher virus transformed lymphoma) or EL4 (H-2^b T cell lymphoma). Vaccinia virus infected target cells were prepared by infection overnight at 5 pfu/cell. Cells were labelled with ⁵¹Cr ($50 \mu\text{Ci}/10^6$ cells) for 60 min, washed and where appropriate, pulsed with $200 \mu\text{M}$ E7.49–57 or NP.366–374 (H-2^b restricted CTL epitope from influenza virus nucleoprotein¹⁰) for 1 h. After further washing, cells were resuspended to $5 \times 10^4/\text{ml}$, and $100 \mu\text{l}$ cell suspension added per well of 96 round bottom well plate.

Six days after restimulation, cells from peptide stimulated cultures were harvested. Three-fold dilutions were prepared, and $100 \mu\text{l}$ added per well to give a range of effector: target (E:T) cell ratios, each tested in triplicate. Control wells were also prepared containing target cells plus medium alone (spontaneous chromium release), and target cells plus 5% Triton X-100 (maximum release). Plates were centrifuged at 1200 r.p.m. for 1 min, and the cells incubated at 37°C for 4 h. At the end of this period, $25 \mu\text{l}$ supernatant was harvested and counted in a Wallac 1450 Microbeta Plus liquid scintillation counter. Specific lysis was determined by the standard formula:

% specific lysis =

$$\frac{\text{sample release cpm} - \text{spontaneous release cpm}}{\text{maximum release cpm} - \text{spontaneous release cpm}}$$

RESULTS

Selection of the recombinant virus vector

Vaccinia virus was chosen as the vector for delivery of the HPV gene sequences for a number of reasons. First, vaccinia virus was used on a massive scale as a vaccine against smallpox; hence a great deal of information is available about its safety in humans. Complications can occur as a result of vaccination³⁰, but these are rare, occurring mainly in children who inadvertently spread the virus to their eyes. Thus for treatment of cervical tumours, where the prognosis can be very poor, the potential benefits of this approach clearly outweigh the risks. The genome of vaccinia virus can be readily manipulated to incorporate additional DNA³¹, and infection with recombinant vaccinia viruses expressing heterologous antigens induces a variety of immune responses, including CTL, in both experimental animals and humans³². Finally recombinant vaccinia viruses expressing a variety of tumour-associated antigens, including those encoded by papillomaviruses, have been used successfully to provide protection against tumour growth in animal models^{14,33–35}.

Vaccinia virus also provides certain particular advantages for delivery of HPV E6 and E7 genes. These genes are believed to contribute to the oncogenic process, and to be able to immortalise cells in culture. It is important therefore to consider the likelihood that vaccination with the recombinant virus could lead to long term expression of E6 and E7 that could result in cell

transformation. Since vaccinia is a lytic virus that replicates in the cell cytoplasm and does not establish a state of persistence or latency, the possibility that a cell infected with a recombinant expressing E6 and E7 could survive to become tumorigenic is extremely small. Furthermore, the expression of heterologous DNA from the vaccinia genome is dependent upon specialist vaccinia promoters which differ substantially from normal mammalian cell promoters, and which rely on vaccinia-encoded transcription components. It is therefore highly unlikely that DNA released from the recombinant vaccinia virus infected cells could be taken up by healthy cells leading to long term gene expression is also remote.

We chose the Wyeth vaccine strain of vaccinia for construction of the HPV-recombinant virus, on the basis that among the various strains used during the smallpox vaccination campaign, it was associated with the lowest level of complications³⁰.

Selection and modification of HPV genes for incorporation into the recombinant virus

We considered that both the E6 and E7 proteins were valid targets for raising anti-tumour responses. Furthermore, since the majority of tumours contain DNA from either HPV16 or HPV18, we elected to include the E6 and E7 coding sequences from both virus types. To simplify the problem of expressing four different heterologous coding sequences within a single recombinant vaccinia virus, the E6 and E7 coding sequences were fused together by site-directed mutagenesis to create a single open reading frame for each virus type. Since the E7 gene lies immediately downstream of E6 on the genome of both HPV16 and HPV18, this was achieved by replacement of the E6 termination triplet with a four nucleotide sequence that removed the stop codon, and aligned the two reading frames. This created a simple fusion of the two coding sequences linked by the amino acid sequence Gly-Ile, for the HPV16 E6/E7 sequence, and Gly-Ile-Leu-Ser in the case of the HPV18 E6/E7 sequence [Figure 1(a)].

We noted that the open reading frame for HPV18 E6 contained the sequence TTTTATTCTAGAATAGAG (at position 210 from the start of E6). This sequence contains the motif TTTTNT which could act as termination signal for vaccinia transcription³⁶, and is also a region of particularly close similarity with the HPV16 E6 coding sequence. We therefore modified this region by site-directed mutagenesis to avoid the potential for improper gene expression, and for recombination between the two sets of homologous sequences within the recombinant virus genome [Figure 1(c)]. This was done without altering the amino acid coding sequence.

Although the potential risk of oncogenesis following infection with a recombinant vaccinia expressing the E6/E7 sequences is undoubtedly extremely low, we created a further margin of safety by altering the sequences to reduce their capacity for cell immortalisation. This was achieved by site directed mutagenesis of the E7 coding sequences in a manner predicted to abolish binding to cellular Rb protein [Figure 2(a)]. Thus for HPV16 E7, the codons for Glu24 and Cys26, which are known to be crucial for Rb binding, and which are conserved in a number of different Rb binding proteins³⁷ were altered to glycine codons. Likewise in HPV18, the equivalent codons, Glu27 and Cys29 were altered to glycine codons.

Table 1 Abolition of co-transformation activity by modification of HPV16 E6/E7 genes

Test plasmid	Number of colonies	
	pJ3	pJ4
No insert	0	0
Wild type E7	132	104
E6/E7 fusion	9	17
E6/E7 fusion (modified E7)	0	0

We confirmed that the changes introduced into the E7 coding sequences had indeed abolished Rb binding using a previously published procedure²⁰. The wild type E7 and modified E7 coding sequences of both the HPV16 and HPV18 were transcribed and translated *in vitro* in the presence of radiolabelled amino acids. The extracts were then mixed with unlabelled *in vitro* translated cellular Rb protein, and binding assessed by immunoprecipitation of radiolabelled HPV proteins using an anti-Rb monoclonal antibody [Figure 2(b)]. The presence of bands corresponding to the wild type HPV16 and HPV18 E7 proteins in lanes 1 and 3 respectively confirmed their capacity to bind Rb. No binding could be detected, however, using the E7 proteins carrying the Rb binding site modifications (lanes 2 and 4).

In a further experiment we assessed the capacity of the altered HPV16 E6/E7 coding sequences to mediate transformation of primary rat embryo fibroblasts using an assay based on co-transformation with an activated Ras oncogene²¹. The modified and unmodified forms of the E6/E7 sequences were cloned into plasmids adjacent to the MoLV LTR or the SV40 promoter and their co-transformation capacities compared (Table 1). Transformation with wild type E7 driven by either promoter produced a substantial number of immortalised colonies (104 and 132, respectively). The number of colonies generated by transformation with the fused, but unmodified E6/E7 sequences was, however, substantially less (17 and 9). As expected, no colonies were observed where the E6/E7 sequences had been modified to eliminate Rb binding.

These experiments confirmed that the modified HPV sequences to be inserted into vaccinia virus had substantially reduced capacity for cell transformation.

Insertion of HPV sequences into vaccinia virus

We considered that the level of safety associated with the use of the Wyeth strain of vaccinia virus³⁰ was sufficient to justify vaccination of cervical cancer patients, and so our strategy for insertion of HPV sequences into the Wyeth strain was to select on the basis of nucleotide sequence analysis, a site within the genome whose disruption was unlikely to affect the capacity of the virus to replicate and hence to generate an immune response. Figure 3 shows the site selected for insertion. It lies within a region of the virus genome defined by the restriction enzyme fragment SalIF and represents an intergenic region between the SalIF15R and SalIF16R open reading frames (ORFs)³⁸.

To provide a recombination vehicle for insertion of the HPV sequences into the vaccinia genome, plasmid pIMMB7a was constructed, which contained a 1kb fragment spanning symmetrically the chosen insertion site within the vaccinia virus genome (located in the SalI F fragment, and defined by the sequence CTACCAG

ATTAT –insert–TATGTGTTATAT). The fragment was prepared directly from vaccinia DNA by PCR. pIMMB7a also contained the guanine phosphoribosyl transferase (gpt) gene under the control of the vaccinia 7.5K promoter, and the f1 phage origin of replication. This plasmid was modified by site directed mutagenesis to incorporate a unique SnaB I site at the precise point at which DNA was to be inserted (pIMMB8a). Plasmid pIMMB9a (Figure 4) was obtained by insertion at the SnaB I site of a cassette containing the HPV16 and HPV18 coding sequences inserted at the same site and inverted with respect to each other. The coding sequences for the HPV16 E6/E7 fusion (from pIMS7.2) and HPV18 E6/E7 fusion (from pIMS8.2) were placed under the control of the vaccinia virus specific promoters H6 and p7.5 respectively^{25,26}. These were selected on the basis that they are known to operate at early as well as late times during virus infection; there have been reports that genes expressed only late in the vaccinia virus infection cycle fail to provoke MHC class I-restricted CTL responses³⁹.

We used a two-stage “transient dominant” selection procedure²⁷ to insert the HPV gene cassette into the Wyeth vaccinia virus genome in a manner that did not result in the retention of any unwanted sequences (Figure 4). During the first stage recombinant viruses which had acquired by homologous recombination the whole of the plasmid pIMMB9a were selected on the basis of their resistance to mycophenolic acid. The second stage involved passage of this virus in the absence of mycophenolic acid. Internal recombination between homologous sequences within the genome then resulted in formation of either wild type virus once more (designated vwt.1), or a recombinant virus containing only the desired HPV cassette. The latter virus, v9a.1 (subsequently designated TA-HPV) was cloned by repeated plaque purification and limiting dilution and characterised extensively.

Characterisation of TA-HPV

Genetic analysis. PCR analysis was used to confirm the presence of the HPV gene cassette within the recombinant virus. This analysis is shown in Figure 5. Pairs of primers chosen to bind outside and inside the insertion yielded DNA fragments of precisely the size expected. We further characterised the gene insert by direct nucleotide sequence analysis of purified virus DNA using a PCR-based “cycle sequencing” procedure, and comparison with sequences deduced before insertion revealed no changes. Further experiments were carried out to confirm the genetic stability of the insert during multiple passage of the recombinant virus at both high and low multiplicities of infection. Following nine serial passages in MRC-5 cells, 20 individual plaque isolates were prepared and tested for the presence of the HPV expression cassette by PCR as described above; all showed the presence of the expected DNA insert (data not shown).

Expression of HPV proteins

We wished to confirm that the recombinant virus was capable of expressing the expected gene products following infection. Infected cells were therefore analysed by western blotting using antibodies specific for the HPV16

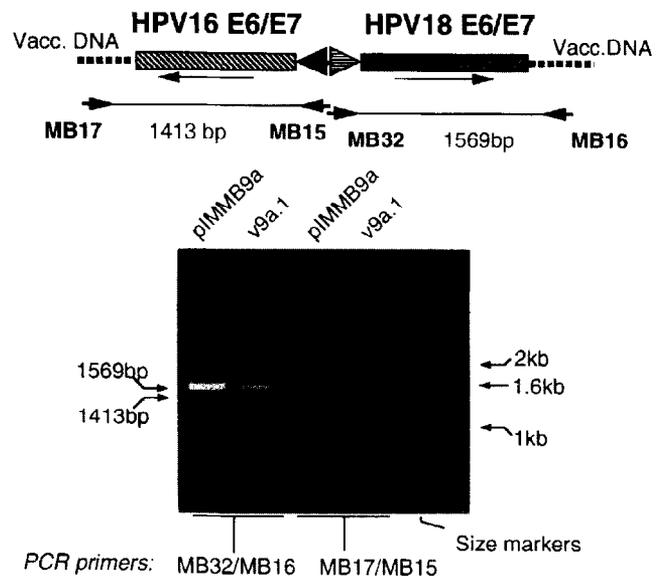


Figure 5 PCR analysis of the HPV sequences in v9a.1. The expected orientation of the inserted HPV sequences is shown, along with the location of the PCR primers, and the expected size of the reaction products. The results of the amplification from v9a.1 as well as control plasmid pIMMB9a are shown.

E7 or the HPV18 E7 proteins. Figure 6 shows that v9a.1 produced proteins of the size expected for the HPV16 and HPV18 E6/E7 proteins, as judged by co-migration with equivalent *in vitro* translated markers, whereas these proteins were not produced by the control Wyeth virus.

Comparative virulence analysis

The strategy for insertion of HPV-specific information into the Wyeth virus genome was designed to create a recombinant virus whose growth properties and hence capacity to provoke an immune response were similar to the parent virus. To test this, we compared the growth properties of v9a.1 with Wyeth *in vivo* in a mouse model. Figure 7(a) shows that the titres of the two viruses detectable in mouse lungs were similar at day 1 following intra-nasal infection, although lower titres of v9a.1 were obtained at days 3 and 5; each virus was cleared from the lungs within 14 days of infection. These data suggest that insertion of the HPV gene cassette into the Wyeth genome did not substantially compromise virus growth. Comparison of the two viruses by intracranial inoculation however, indicated that the recombinant virus was substantially less neurovirulent than the parent Wyeth strain [Figure 7(b)]; at the highest dose used (10^7 pfu), only 1/12 deaths occurred with v9a.1, whereas 10/12 of the Wyeth inoculated animals died. This difference in virulence was reproduced when immunosuppressed “nude” mice were infected with the two viruses (data not shown); once again the severity of disease (as determined by death, presence of vaccinia lesions and weight loss) was much greater for the Wyeth parent than for the recombinant virus. These results were unexpected, and so we compared the neurovirulence of the Wyeth strain directly with a “sibling” virus to TA-HPV, whose genetic composition should be identical except for the presence of the HPV expression cassette. This virus, designated “vwt.1” was generated during the second round of recombination to generate TA-HPV (Figure 4). This experiment indicated that

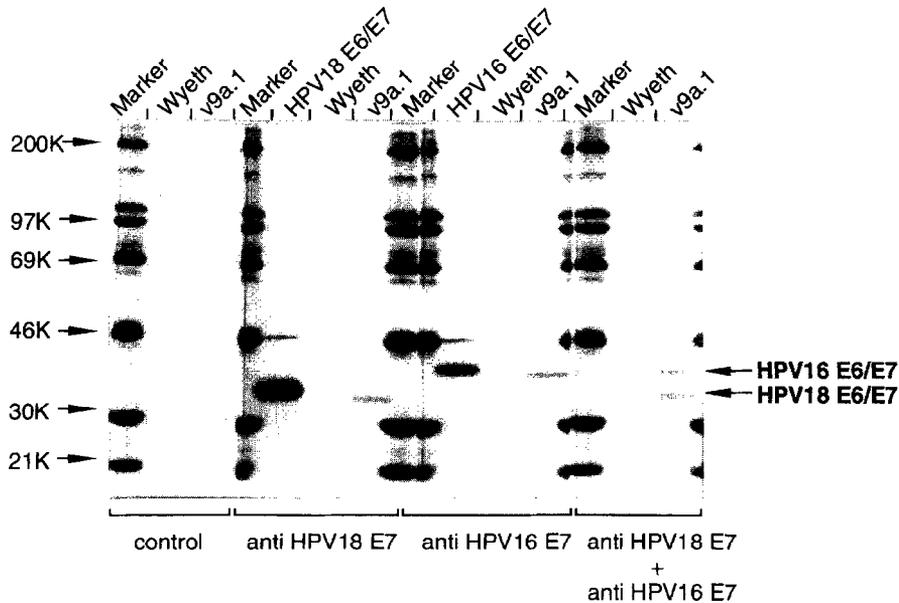


Figure 6 Western blot analysis of lysates of MRC-5 cells infected with v9a.1 or Wyeth. Lanes 1, 4, 8 and 12 contained radiolabelled molecular weight markers. Lanes 6 and 7 were probed with anti-HPV18 E7 (E710). Lanes 9 and 10 were probed with anti-HPV16 E7 (Camvir 3). Lanes 13 and 14 were probed with Camvir 3 and E710 in combination. An antibody against HPV18 L1 (F1/1.1.16) was used as a negative control (lanes 2 and 3). Radiolabelled HPV18 and 16 E6/E7 fusion proteins produced by *in vitro* transcription and translation were run as markers in lanes 5 and 9 respectively.

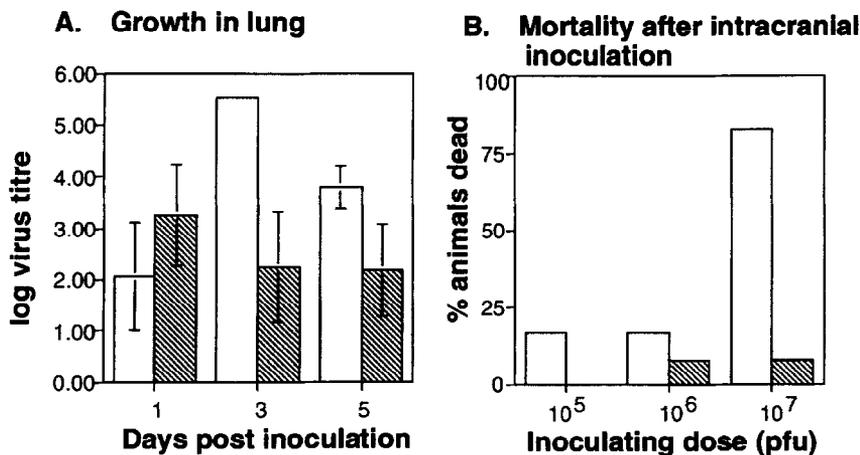


Figure 7 (A) Comparative growth of TA-HPV (hatched columns) and parental Wyeth vaccinia virus (blank columns) in mouse lungs. Groups of 18 mice were inoculated intranasally with 106.7pfu of virus. At each time point, lungs were taken from six animals, homogenised and the virus present was titrated by plaque assay. Error bars represent the standard error of the logarithmic mean. (B) Comparative neurovirulence of TA-HPV and Wyeth virus in mice. Groups of 12 animals were injected intracranially with 50 μ l containing varying doses of TA-HPV (hatched columns) or parental Wyeth vaccinia virus (blank columns). Survival of the animals was monitored twice daily for 21 days.

vwt.1 also had reduced neurovirulence properties after intra-cranial inoculation with respect to Wyeth (data not shown) suggesting that the difference was not due to the insertion of the HPV gene cassette, but rather reflected heterogeneity in the original Wyeth stock.

Induction of HPV-specific CTL in mice by immunisation with TA-HPV

We wished to confirm that infection of mice with TA-HPV could stimulate a CTL response against the expressed HPV antigens, and that these cells could recognise target cells expressing HPV antigens. C57BL/6 mice were inoculated three times intra-peritoneally with TA-HPV, or Wyeth vaccinia, or vE710 (a recombinant vaccinia virus expressing the unmodified HPV16 E7 sequence) and spleens were removed 9 days after the final immunisation. Spleen cells were cultured *in vitro* in

the presence of a synthetic peptide corresponding to a known epitope from HPV16 E7 (E7.49–57) for H-2^b restricted CTL in C57BL/6 mice¹³. After 12 days in culture the resulting cell lines were tested for their ability to kill target cells pulsed with the HPV16 E7.49–57 peptide. Cell lines derived from TA-HPV or vE710 mice lysed histocompatible target cells (RMA) pulsed with the E7 49–57 peptide at equivalent levels, but did not lyse unpulsed target cells, or cells pulsed with the H-2^b restricted CTL epitope from influenza virus NP.366–374. There was no lysis of any of the target cells by lines derived from spleens from unimmunised or Wyeth immunised mice (Figure 8a). After culture for an additional 2 weeks, cell lines derived from the TA-HPV immunised mice were tested for their ability to recognise both peptide pulsed target cells, and also cells expressing HPV16 E7 following infection with recombinant vaccinia viruses (Figure 8b). There was

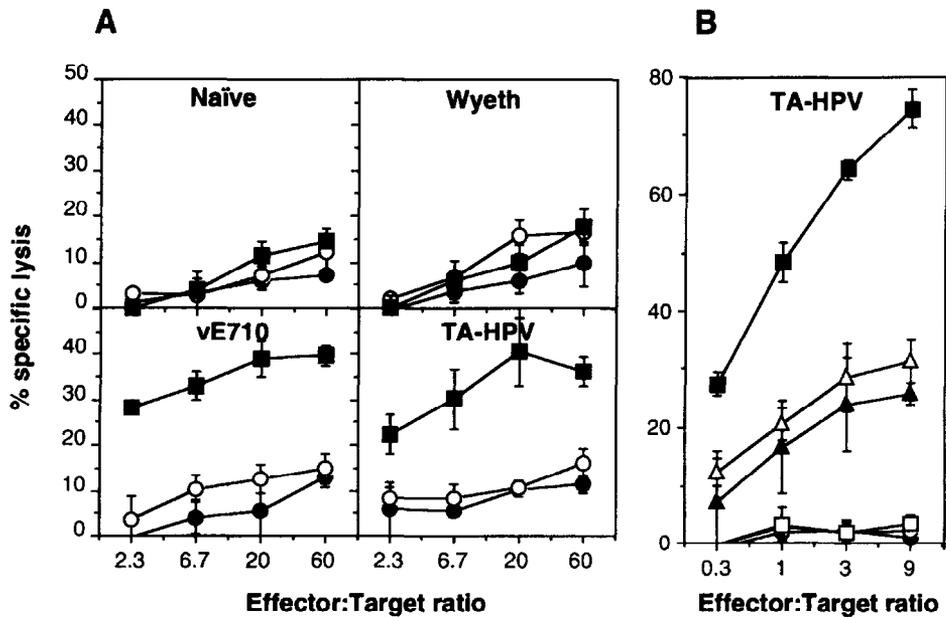


Figure 8 Induction of CTL by TA-HPV. (A) Cytotoxic activity of spleen cells from C57BL/6 mice immunised with PBS, Wyeth strain, vE710, or TA-HPV after 12 days stimulation *in vitro* with 10 μ M synthetic peptide corresponding to HPV16 E7.49-57. Target cells were RMA (H-2b), unpulsed (closed circles), pulsed with flu NP.366-374 (open circles) or pulsed with E7.49-57 (closed squares). (B) Cytotoxic activity of spleen cell cultures from TA-HPV immunised mice after 27 days in culture in the presence of E7.49-57. Peptide pulsed target cells were the same as in panel A. In addition RMA cells infected with Wyeth strain (open squares), vE710 (closed triangles) and TA-HPV (open triangles) were also tested.

significant lysis of EL4 cells infected with TA-HPV or vE710, indicating that the CTLs generated by TA-HPV were capable of recognising the wild type HPV16 E7 sequence, after synthesis and processing within the target cells. Lysis of TA-HPV and vE710 infected target cells was less than that of cells pulsed with the E7.49-57 pulsed targets, probably as a result of a reduced epitope density at the cell surface, compared to peptide-pulsed target cells. There was no lysis of target cells infected with Wyeth strain vaccinia virus. In addition, CTL generated by immunisation with vE710 were capable of lysing target cells infected with TA-HPV (data not shown).

DISCUSSION

We have designed and constructed a recombinant vaccinia virus expressing the HPV 16 and HPV 18 E6 and E7 proteins specifically for use in the treatment of human cervical cancer. The parent virus on which the construct was based is a well-characterised vaccine strain of vaccinia virus with an extensive history of use in humans. The recombinant HPV sequences inserted were modified to increase the safety margin likely to be associated with its use in humans. We showed that the recombinant virus carried HPV DNA in the expected configuration, and that the heterologous coding sequences were expressed successfully as protein products in infected cells. Animal model studies indicated that the recombinant virus had reduced neurovirulence properties compared to the parental Wyeth strain. Although intra-nasal inoculation of the recombinant produced somewhat lower titres than the parental virus, vaccination of mice with v9a.1 led to the generation of HPV-specific CTLs.

There are a number of reports from both animal models and human studies that highlight CTLs in particular as effective agents for tumour cell

destruction^{11,12,40}, although other types of immune response may also play a role. These studies have also shown that effective anti-tumour responses can be induced by vaccination even where the tumour cells themselves are poorly immunogenic. The goal of our work has been to develop an agent that can induce an aggressive anti-tumour response at the time of primary cervical cancer diagnosis to help clear residual tumour cells that remain after conventional therapy (surgery or radiotherapy). Success at this stage should help prevent or delay disease recurrence, and since recurrence is almost always fatal within a relatively short time, this would represent a major clinical advance. To this end we have recently carried out an initial clinical study in cervical cancer patients with the virus described in this report. The results of this study, which was designed primarily to evaluate the safety of the approach, and to assess HPV specific immune responses, in particular CTL activity, have been reported separately (Borysiewicz *et al.*, submitted for publication).

Note added in proof:

Borysiewicz *et al.*, submitted for publication is now: Borysiewicz, L.K., Fiander, A., Nimako, M., Man, S., Wilkinson, G.W.G., Westmoreland, A.S., Evans, M., Stacy, S.N., Boursnell, M.E.G., Rutherford, E., Hickling, J.K. and Ingis, S.C. A recombinant vaccine virus encoding human papillomavirus types 16 and 18, E6 and E7 proteins as immunotherapy for cervical cancer. *Lancet* 1996, **347**, 1523-1527.

ACKNOWLEDGEMENTS

The authors wish to thank Professor G.L. Smith for providing vaccinia virus sequence data, and for advice on the construction of the recombinant v9a.1. We also wish to thank Tony Churchill PhD and Julie Churchill

for assistance with the *in vivo* virulence studies and CTL experiments.

REFERENCES

- 1 zur Hausen, H. Viruses in human cancers. *Science* 1991, **254**, 1167–1173
- 2 van den Brule, A.J.C., Snijders, P.J.F., Meijer, C.J.L.M. and Walboomers, J.M.M. PCR based detection of genital HPV genotypes: An update and future perspectives. *Papillomavirus Reports* 1993, **4**, 95–99
- 3 Schwartz, E., Freese, U.K., Gissman, L. et al. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature* 1985, **314**, 111–114
- 4 Hawley-Nelson, P., Vousden, K.H., Hubbert, N.L., Lowy, D.R. and Schiller, J.T. HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *EMBO J* 1989, **8**, 3905–3910
- 5 Phelps, W.C., Yee, C.L., Munger, K. and Howley, P.M. The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. *Cell* 1988, **53**, 539–547
- 6 Dyson, N., Howley, P.M., Munger, K. and Harlow, E. The human papillomavirus type 16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* 1989, **243**, 934–937
- 7 Munger, K., Werness, B.A., Dyson, N., Phelps, W.C., Harlow, E. and Howley, P.M. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumour suppressor gene product. *EMBO J* 1989, **8**, 4099–4105
- 8 Werness, B.A., Levine, A.J. and Howley, P.M. Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* 1990, **248**, 76–79
- 9 von Knebel Doeberitz, M., Ritmuller, C., Aengeneyndt, P., Jansendurr, D. and Spitovsky, D. Reversible repression of papillomavirus oncogene expression in cervical carcinoma cells. Consequences for the phenotype and E6-p53 and E7-pRb interactions. *J Virol* 1994, **68**, 2811–2821
- 10 Townsend, A.R.M., Rothbard, J.B., Gotch, F.M., Bahadur, G., Wraith, D. and McMichael, A.J. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* 1986, **44**, 959–968
- 11 Rooney, C.M., Smith, C.A., Ng, C.Y.C. et al. Use of gene modified virus specific T lymphocytes to control Epstein-Barr virus related lymphoproliferation. *Lancet* 1995, **345**, 9–13
- 12 Kawakami, Y., Elyahu, S., Delgado, C.H. et al. Identification of a human melanoma antigen recognized by tumour infiltrating lymphocytes associated with *in vivo* tumour rejection. *Proc Natl Acad Sci (USA)* 1994, **91**, 6458–6462
- 13 Feltkamp, M.C.W., Smits, H.L., Vierboom, M.P.M. et al. Vaccination with cytotoxic T lymphocyte epitope containing peptide protects against a tumor induced by human papillomavirus type 16 cells. *Eur J Immunol* 1993, **23**, 2242–2249
- 14 Meneguzzi, G., Cerni, C., Kieny, M.P. and Lathe, R. Immunisation against human papillomavirus type 16 tumour cells with recombinant vaccinia viruses expressing E6 and E7. *Virology* 1991, **181**, 62–69
- 15 Chen, L.P., Thomas, E.K., Hu, S.L., Hellstrom, I. and Hellstrom, K.E. Human papillomavirus type 16 nucleoprotein E7 is a tumour rejection antigen. *Proc Natl Acad Sci (USA)* 1991, **88**, 110–114
- 16 Chen, L.P., Mizuno, M.T., Singhal, M.C. et al. Induction of cytotoxic T lymphocytes specific for a syngeneic tumour expressing the E6 oncoprotein of human papillomavirus type 16. *J Immunol* 1992, **148**, 2617–2621
- 17 Barrary G. and Merrifield R. Solid phase peptide synthesis. In: *The Peptides* (Eds. Gross E. and Meienhofer J.), New York: Academic Press, 1979, 1–284
- 18 Cole, S.T. and Danos, O. Nucleotide sequence and comparative analysis of the human papillomavirus type 18 genome. *J Mol Biol* 1987, **193**, 599–608
- 19 Brierley, I., Digard, P. and Inglis, S.C. Characterisation of an efficient coronavirus ribosomal frameshifting signal: Requirement for an RNA pseudoknot. *Cell* 1989, **57**, 537–547
- 20 Barbosa, M.S., Edmonds, C., Fisher, C., Schiller, J.T., Lowy, D.R. and Vousden, K.H. The region of the HPV E7 oncoprotein homologous to adenovirus E1a and SV40 large T antigen contains separate domains for Rb binding and casein kinase II phosphorylation. *EMBO J* 1990, **9**, 153–160
- 21 Chesters, P.M., Edmonds, C., Vousden, K.H. and McCance, D.J. Analysis of human papillomavirus type 16 open reading frame E7 immortalizing function in rat embryo fibroblast cells. *J Gen Virol* 1990, **71**, 449–453
- 22 Morgenstern, J.P. and Land, H.D. A series of mammalian expression vectors and characterisation of their expression of a reporter gene in stably and transiently transfected cells. *Nucleic Acids Res* 1990, **18**, 1068–1072
- 23 Storey, A., Pim, D., Murray, A., Osborn, K., Banks, L. and Crawford, L. Comparison of the *in vitro* transforming activities of human papillomavirus types. *EMBO J* 1988, **7**, 1815–1820
- 24 Shih, C. and Weinberg, R.A. Isolation of a transforming sequence from a human carcinoma cell line. *Cell* 1982, **29**, 161–169
- 25 Venkatesan, S., Baroudy, B.M. and Moss, B. Distinctive nucleotide sequences adjacent to multiple initiation and termination sites of an early vaccinia virus gene. *Cell* 1981, **125**, 805–813
- 26 Rosel, J.L., Earl, P.L., Weir, J.P. and Moss, B. Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late genes deduced by structural and functional analysis of the Hind III H genome fragment. *J Virol* 1986, **60**, 436–449
- 27 Falkner, F.G. and Moss, B. Transient dominant selection of recombinant vaccinia viruses. *J Virol* 1990, **64**, 3108–3111
- 28 Stacey, S.N., Eklund, C., Jordan, D. et al. Scanning the structure and antigenicity of HPV16 E6 and E7 oncoproteins using anti-peptide antibodies. *Oncogene* 1994, **9**, 635–645
- 29 Sadovnikova, E., Zhu, X., Collins, S.M. et al. Limitations of predictive motifs revealed by cytotoxic T lymphocyte epitope mapping of the human papillomavirus E7 protein. *Int Immunol* 1994, **6**, 289–296
- 30 Lane, J.M., Ruben, F.L., Neff, J.M. and Milar, J.D. Complications of smallpox vaccination 1968. National surveillance in the United States. *New Engl J Med* 1969, **281**, 1201–1208
- 31 Moss, B. and Flexner, C.H. Vaccinia virus expression vectors. *Annu Rev Immunol* 1987, **5**, 305–324
- 32 Bennink, J.R. and Yewdell, J.W. Recombinant vaccinia viruses as vectors for studying T lymphocyte specificity and function. *Curr Top Microbiol Immunol* 1990, **163**, 153–184
- 33 Estin, C.D., Stevenson, U.S., Plowman, G.D. et al. Recombinant vaccinia virus vaccine against the human melanoma antigen p97 for use in immunotherapy. *Proc Natl Acad Sci (USA)* 1988, **85**, 1052–1056
- 34 Hareuveni, M., Gautier, C., Kieny, M.-P., Wreschner, D., Chambon, P. and Lathe, R. Vaccination against tumour cells expressing breast cancer epithelial cell antigen. *Proc Natl Acad Sci (USA)* 1990, **87**, 9498–9502
- 35 Kantor, J., Irvine, K., Abrams, S., Kaufmann, H., DiPietro, J. and Schlom, J. Anti-tumour activity and immune responses induced by a recombinant carcinoembryonic antigen-vaccinia virus vaccine. *J Natl Cancer Inst* 1992, **84**, 1084–1091
- 36 Rohrmann, G., Yuen, L. and Moss, B. Transcription of vaccinia virus early genes by enzymes isolated from vaccinia virions terminates downstream of a regulatory sequence. *Cell* 1986, **46**, 1029–1035
- 37 Edmonds, C. and Vousden, K.H. A point mutational analysis of human papillomavirus type 16 E7 protein. *J Virol* 1989, **63**, 2650–2656
- 38 Smith, G.L., Chan, Y.S. and Howard, S.T. Nucleotide sequence of 42kbp of vaccinia virus strain WR from near the right inverted terminal repeat. *J Gen Virol* 1991, **72**, 1349–1376
- 39 Andrew, M.E., Coupar, B.E., Boyle, D.B. and Blanden, R.V. Recognition by major histocompatibility class I restricted cytolytic T lymphocytes of vaccinia encoded viral and class I proteins. *Eur J Immunol* 1987, **17**, 1515–1518
- 40 Schendel, D.J., Gansbacher, B., Oberneder, R. et al. Tumour specific lysis of human renal cell carcinomas by tumour infiltrating lymphocytes. *J Immunol* 1993, **151**, 4209–4220