J Clin Pathol 1990;43:488–492

Production and characterisation of a monoclonal antibody to human papillomavirus type 16 using recombinant vaccinia virus

C S McLean, M J Churcher, J Meinke, G L Smith, G Higgins, M Stanley, A C Minson

Abstract

A monoclonal antibody was raised against the major capsid protein L1 of human papillomavirus type 16, using a recombinant vaccinia virus that expresses the L1 protein, as a target for screening. This antibody, designated CAMVIR-1, reacted with a 56 kilodalton protein in cells infected with L1-vaccinia virus, and the protein was present in a predominantly nuclear location. The antibody also detects the HPV-16 L1 antigen in formalin fixed, paraffin wax embedded biopsy specimens and on routine cervical smears. The antibody reacts strongly and consistently with biopsy specimens containing HPV-16 or HPV-33, but very weak reactions were occasionally observed with biopsy specimens or smears containing HPV-6 or HPV-11.

The potential advantages of using a vaccinia recombinant are (i) the target protein is synthesised in a eukoryotic cell so that its "processing" and location are normal; (ii) cells infected with vaccinia recombinants can be subjected to various fixing procedures similar to those used for routine clinical material. This greatly increases the probability that an identified antibody will be useful in a clinical setting.

Department of Pathology, Divisions of Cellular Pathology and Virology, University of Cambridge
C S McLean
M J Churcher
J Meinke
G L Smith
M Stanley
A C Minson

Division of Virology, Institute of Medical and Veterinary Sciences, Adelaide, South Australia
G Higgins

Correspondence to:
Dr C S McLean, Department of Pathology, University of Cambridge, Cambridge CB2 1QP

Accepted for publication 15 February 1990

Human papillomaviruses (HPV) are small DNA viruses which cause a variety of proliferative lesions on epithelial or mucosal surfaces.[1] More than 50 HPV types have now been identified, and several of these are associated with premalignant and malignant disease of the anogenital region. In particular, HPV 6 and 11 have been identified in benign lesions; HPV 16, 18, 31 and 33 are highly significantly associated with all grades of cervical intraepithelial neoplasia (CIN) and cervical cancer.[2,3]

There is no suitable tissue culture system for the propagation of these viruses and the identification of viral DNA and classification of viruses is based on DNA homology using hybridisation techniques.[4–6] Viral DNA sequences have been identified and typed in abnormal cervical lesions and in cervical smears using blot hybridisation techniques, and more recently by the polymerase chain reaction.[7] The exquisite sensitivity of the latter has resulted in the detection of low copy viral DNA in histologically and cytologically normal subjects. The ubiquity of viral DNA highlights the need for reagents that will detect viral proteins. Several groups have recently tried to overcome the problems associated with the lack of a permissive in vitro culture system by cloning and expressing viral genes in prokaryotic and eukaryotic expression systems.[8–12] In an earlier paper we reported the construction of a vaccinia virus recombinant containing the HPV 16 L1 gene (encoding the major capsid antigen), and the preparation of polyclonal antisera raised against a bacterial fusion protein containing HPV 16 L1 sequences.[13] Polyclonal antisera, however, are of limited use in that they cannot give information about the presence of type specific epitopes on the L1 protein and tend to result in high background in immunohistochemical and immunoprecipitation studies. There is a need for type specific reagents for diagnostic as well as for research purposes for viruses such as HPV 16, which has been identified in 50–60% of cases of cervical carcinoma subjected to viral typing.[5]

In this report we describe the production of a monoclonal antibody to HPV 16 L1 using a novel protocol in which viral antigen is generated for immunisation using a bacterial expression system and hybridoma supernants are screened for specific antibody using a vaccinia recombinant expressing the target protein.

Methods

The myeloma cell line NSO and established hybridoma cell lines were maintained in Glasgow modified Eagle's medium (GMEM) containing 5% fetal calf serum (FCS). African green monkey kidney (CV-1) cells were maintained in the same medium containing 10% FCS. Baby hamster kidney 21 clone 13 cells (BHK) were maintained in GMEM containing 10% tryptose phosphate broth and 10% newborn calf serum. The construction of recombinant vaccinia virus (VL1RK) expressing the entire HPV 16 L1 open reading frame under the control of a vaccinia late promoter has been described previously.[13] The recombinant virus and the parent WT vaccinia virus (strain WR) were grown in BHK cells and titrated in CV-1 cells.

MOUSE IMMUNISATION AND CELL FUSION

Mice were immunised subcutaneously with 20 μg of a β galactosidase-L1 fusion protein (containing amino acids 198-C terminus of HPV-16 L1[13]) in complete Freund's adjuvant. After

four weeks a series of subsequent doses of 15 μg protein in Freund's incomplete adjuvant were given intraperitoneally at two weekly intervals. A final intravenous immunisation of 15 μg was given three days before the fusion. The spleen was removed aseptically and fused to the mouse hybridoma NSO, using the original protocol.[14 15] Cells were plated at a density of 2×10^6/well in 24-well Linbro plates, and the growth medium changed every two to three days. Positive hybrids were cloned twice by limiting dilution, and clones secreting antibody to L1 were grown in 75 cm^2 flasks and stored in liquid nitrogen.

SCREENING OF HYBRIDOMA SUPERNATANTS USING ELISA

The supernatants of wells containing hybrids were tested in an ELISA as follows: CV-1 cells were grown in 5 mm wells to confluency and infected with 30 plaque forming units/cell WT or VL1RK vaccinia virus. After 18 hours at 37°C cells were fixed in 5% formaldehyde, 10% sucrose, in phosphate buffered saline (PBS), and air dried. Before use cells were permeabilised with 1% Triton X-100, 10% sucrose in PBS. After blocking with 5% FCS, monolayers were reacted with hybridoma supernatants, followed by incubation with peroxidase-linked protein A, each for 30 minutes at room temperature. The wells were then reacted with O-phenylene diamine and the absorption of the supernatants at 492 nm was measured in an ELISA reader.

IMMUNOFLUORESCENCE

CV-1 cells were grown on coverslips and infected, fixed, and permeabilised as described for the ELISA method. Cells were reacted with hybridoma supernatant and subsequently incubated with an anti-mouse IgG fluorescein conjugate.

WESTERN BLOT ANALYSIS

BHK-cell monolayers, infected with 30 pfu/cell WT or VL1RK vaccinia virus, were harvested in PBS 20 hours after infection, lysed in sample buffer (24 mm TRIS-HCl, pH 6·18; 100 mm dithiothreitol; 2% sodium dodceyl sulphate (SDS); 20% glycerol; 0·02% bromophenol blue), and sonicated. Samples were denatured at 100°C for three minutes, subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and the proteins transferred to nitrocellulose by electroblotting as described previously.[9] After blocking in 5% dried milk powder in PBS for 30 minutes at room temperature, filters were incubated with 1/2 diluted supernatant for one hour at room temperature, washed three times in PBS-1% NP40 and reacted with affinity purified ^{125}I-protein A (Amersham International, UK) at a concentration of 0·1 μCi-ml. The blots were washed again in PBS-1% NP40, dried, and autoradiographed.

IMMUNOCYTOCHEMICAL STUDIES

Formalin fixed, paraffin wax embedded sections were obtained from diagnostic biopsy specimens taken from patients attending a routine colposcopy clinic. Sections were dewaxed and rehydrated through a xylene alcohol series. Before immunostaining with the Vectastain ABC complex (Vector Laboratories, Peterborough, England) sections were incubated in 0·1% trypsin at 37°C for five minutes and quenched in 0·3% H_2O_2 for 15 minutes.

Routine cervical Papanicolau smears were fixed in 5% formaldehyde in PBS for five minutes, washed in PBS, permeabilised in 1% Triton and subsequently treated with trypsin, quenched, and stained in the same way as the cervical sections.

IN SITU HYBRIDISATION

Sections of cervical lesions embedded in paraffin wax were dewaxed in xylene and alcohol and hybridised with probes containing DNA of HPV 16/18, HPV 31/33, or HPV 6/11, using the Vira Type in situ hybridisation kit (Life Technologies Inc, Gaithersburg, Maryland, USA) according to the manufacturer's instructions. In certain cases in situ hybridisation was carried out using ^{35}S-labelled DNA, according to the method described by Gowans *et al.*[16] Briefly, slides were rehydrated, digested with proteinase K and pronase, and treated with acetic anhydride before hybridisation with ^{35}S-labelled HPV-16 DNA. After coating with radiographic emulsion slides were stored for eight days at 4°C and developed, fixed, and stained with haematoxylin.

Results

Mice repeatedly immunised with the β galactosidase-HPV 16 L1 fusion protein developed strong responses to the fusion protein, but the response to the vaccinia recombinant expressing HPV-16 L1 was variable. Some mice also gave high "background" responses to cells infected with wild type vaccinia virus. Spleen cells from mice showing a good differential response in ELISAs to the L1 recombinant vaccinia virus relative to the wild type virus were used in fusions with cell line NSO. The results of a series of three fusions were disappointing in that only two positive hybridoma supernatants were identified by ELISAs on cells infected with VL1RK. Of these, only one retained activity following two rounds of cloning by limiting dilution. This hybridoma was stable to subsequent passage and subcloning and was designated CAMVIR-1.

PROPERTIES OF CAMVIR-1

Tissue culture supernatant from CAMVIR-1 reacted with a 56 kilodalton protein in Western blots of cell lysates infected with VL1RK (fig 1a). A minor species of 64 kilodaltons was also detected. This probably represents a glycosylated form of the protein as it is absent in lysates of cells infected in the presence of tunicamycin, an inhibitor of N-glycosylation. Similar results were reported using a polyclonal rabbit serum raised against an HPV-16 L1 fusion protein.[13] No reactivity was observed against cells infected with wild type vaccinia. The antibody also reacted with the fusion protein used for im-

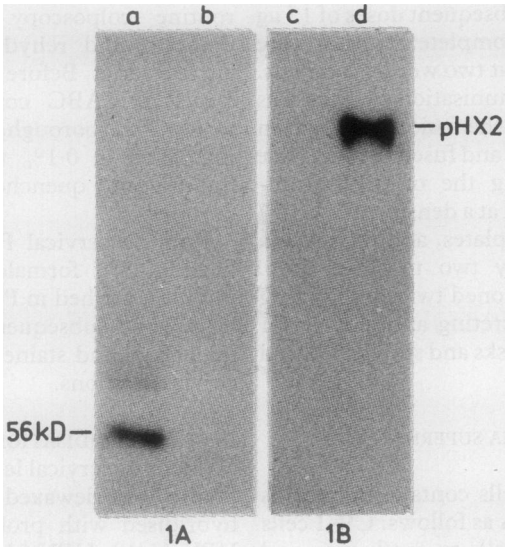

Figure 1 Western blot analysis of reactivity of CAMVIR-1 with L1 fusion protein and L1 vaccinia recombinant. (a) CV1 cells were infected with L1-vaccinia (lane a) or wild type vaccinia (lane b) at 30 pfu/cell, and lysed after 20 hours. Samples were subjected to SDS-PAGE electrophoresis, and after transfer to nitrocellulose the blots were incubated with CAMVIR-1 supernatant diluted at 1/10, followed by affinity purified ^{125}I-protein A at a concentration of 0·1 μCi/ml. After washing, the blots were dried and autoradiographed. (b) 0·1 μg of the purified β-galactosidase L1 fusion protein (pHX2, containing amino acids 221-C terminus (lane d) and 0·1 μg or an irrelevant fusion protein (containing amino acids 1–220 of the HPV-16 L1 protein, (lane c) were subjected to electrophoresis on a 12·5% SDS-acrylamide gel and transferred to nitrocellulose. The blots were reacted with CAMVIR-1 at a dilution of 1/20, followed by peroxidase-linked anti-mouse IgG (Amersham International, UK) at a dilution of 1/200. Bands were visualised using diaminobenzidine tetrahydrochloride (DAB) at a concentration of 1 mg/ml.

munisation but not with an irrelevant fusion protein containing the N-terminal portion of the HPV-16 L1 protein (amino acids 1–197).

To determine the cellular localisation of the antigen detected by CAMVIR-1, cells were infected with recombinant or WT virus and after formaldehyde fixation reacted with CAMVIR-1 and fluorescein-labelled anti-mouse immunoglobulin. In cells infected with L1 vaccinia strong nuclear fluorescence was observed, while no reaction could be detected in cell monolayers infected with WT virus (figs 2g and h). An irrelevant monoclonal antibody of the same subclass (IgG2a) did not react with the recombinant infected cells.

REACTIVITY OF CAMVIR-1 ON TISSUE SECTIONS

The ability of CAMVIR-1 to detect the L1 protein in formalin fixed, paraffin wax embedded tissue sections was examined using a biotin-streptavidin-peroxidase staining method. Sections were obtained from the cervical biopsy specimens of patients with abnormal cervical smears, attending a routine clinic for colposcopy and examination. These lesions were histologically graded as CIN I to III. The sections were tested for the presence of L1 protein using CAMVIR-1 tissue culture supernatant at a concentration of 1/100. Figure 2a shows a lesion in which the nuclei of several cells in the superficial layers are strongly stained. These cells have the morphology of koilocytes. A parallel section of this lesion was subjected to in situ hybridisation to establish the presence and type of viral DNA (fig 2b). The cells with the maximum copy number of HPV 16 DNA are found in the same region of the section as the cells containing L1 protein—that is, in the superficial layers of the lesion. We examined a substantial number of lesions (n = 50), and in those cases where staining with CAMVIR-1 was observed, in situ hybridisation showed high copy numbers of HPV-16 DNA in that region of the section. The opposite was not true, however—that is, a minority of lesions were positive for HPV-16 by in situ hybridisation but showed no staining with CAMVIR-1. In one instance we found that a lesion staining positive with CAMVIR-1 contained HPV31/33DNA. In most cases lesions containing HPV 6 or HPV 11 did not stain with CAMVIR-1, but occasionally a few cells in the superficial layers of these sections would show nuclear staining. We also compared the sensitivity of CAMVIR-1 in detecting L1 protein to that of a commercially available anti-BPV-1-antibody (Dako). On sections positive for HPV-16 or -33 little or no staining was obtained with the Dako antibody, although CAMVIR-1 gave a strongly positive nuclear reaction (figs 2c and d).

DETECTION OF L1 PROTEIN IN CERVICAL SMEARS

The ability of CAMVIR-1 to detect L1 protein in routine cervical smears was investigated in a blind trial. Samples were taken from 50 general practice patients. DNA extractions were performed on exfoliated cells which were subjected to dot blot hybridisation with probes specific for HPV 6, 11, 16 or 18. The smears were examined cytologically and stained with CAMVIR-1 tissue culture supernatant at a 1/100 dilution using the biotin-streptavidin-peroxidase system. The cells were fixed briefly in 5% formaldehyde in PBS, and trypsin was added before reaction. In 14 of the 50 cases an insufficient number of cells was present on the smear to determine if positively stained cells were present. Of the remaining 36 cases, nine contained cells showing nuclear staining and were of koilocytic morphology (figs 2e and f). Decoding showed that two of these contained HPV-16 DNA by dot blot hybridisation (between 2×10^5–10^6 genomic copies). In four cases no HPV DNA could be detected (less than 10^5 genomic copies). In one case HPV DNA was present at low copy number but it was not possible to determine which type. In two cases where positive staining with CAMVIR-1 was observed a very high copy number of HPV-11 was present (more than 10^6 genomic copies a cell). In three cases hybridisation showed the presence of HPV-16 DNA, but no staining with CAMVIR-1 was observed because the smear was inadequate.

Discussion

The absence of a suitable in vitro system for the propagation of human papillomaviruses, together with the fact that in clinical lesions only very low levels of virus are present, has necessitated the use of alternative strategies for the production of immunological reagents to these viruses. In the production of monoclonal antibodies suitable targets for the screening of hybridoma fluids are an important requirement. To date, the monoclonal antibodies reported have been raised and screened against bacterial derived proteins.[16–18] We used cells infected with a vaccinia virus recombinant expressing the HPV 16 L1 open reading frame as a target for the selection of monoclonal antibodies. This system provided a very convenient and plentiful source of viral protein, free of potentially crossreactive bacterial elements.

The antibody against the HPV-16 L1 pro-

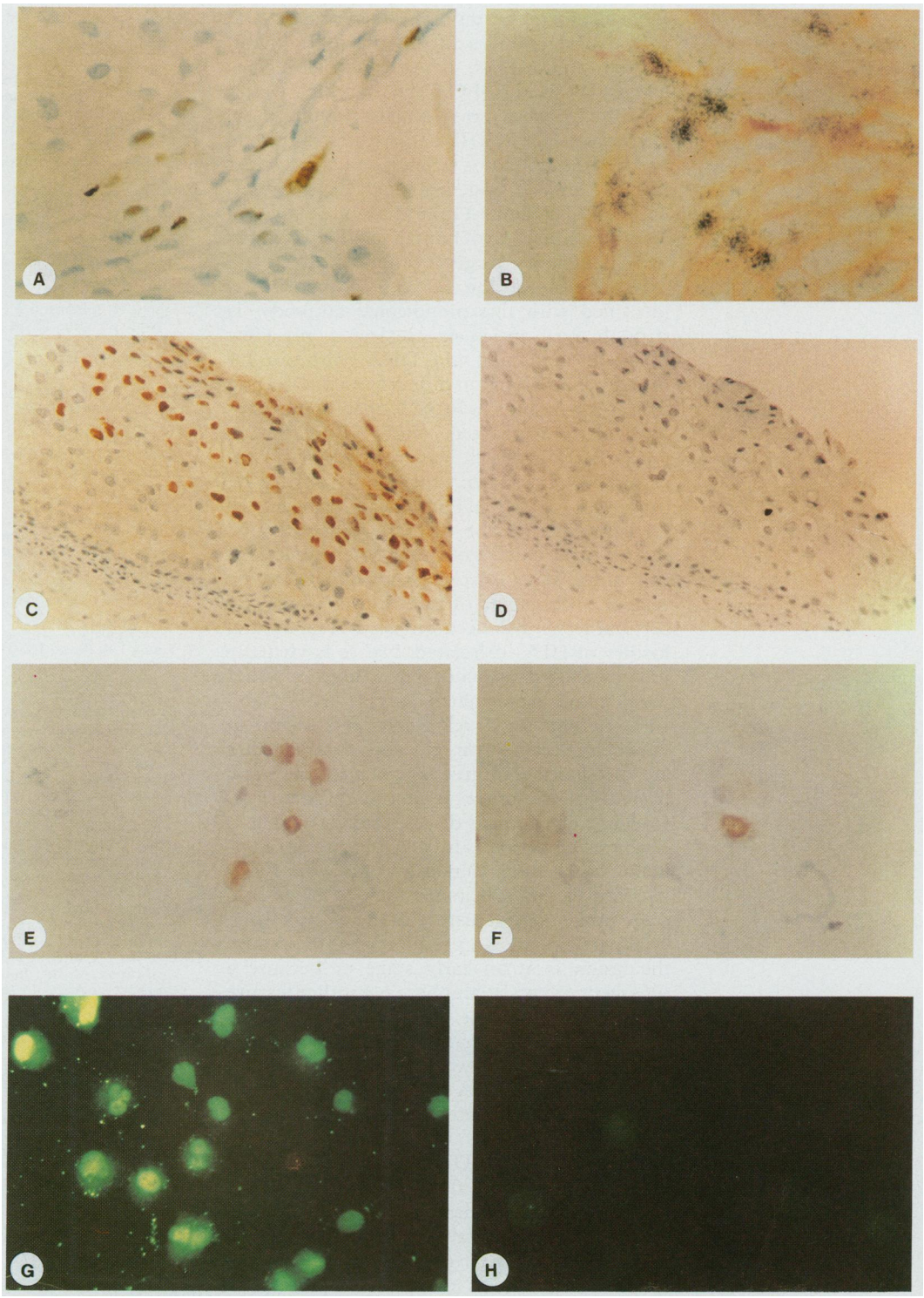

Figure 2 Immunocytochemical staining with CAMVIR-1 of sections of cervical biopsy specimens containing HPV-16 or HPV-33, routine cervical smears, and cells infected with the L1 vaccinia recombinants. (a) Staining with CAMVIR-1 at 1/100 dilution on formol fixed, paraffin wax embedded section of a cervical lesion containing HPV-16. Sections were dewaxed and rehydrated through a xylene alcohol series, incubated in 0·1% trypsin at 37°C for five minutes, quenched in 0·3% H_2O_2 for 15 minutes, and stained using the Vectastain ABC complex. (b) In situ hybridisation using ^{35}S-labelled total HPV-16 DNA on the section adjacent to that in fig 2a. (c) Staining with CAMVIR-1 at 1/100 on a cervical lesion containing HPV-33 using the same protocol as in fig 2a. (d) Staining of the same lesion as in fig 2c, using a commercially available polyclonal anti-BPV-antibody (Dako) at 1/100, followed by the Vectastain ABC complex. (e and f) Staining with CAMVIR-1 at 1/100 of routine cervical smears fixed in 5% formaldehyde-PBS for five minutes, permeabilised in 1% Triton, and subsequently treated with trypsin, quenched, and stained in the same way as the cervical sections. (g and h) Reactivity of CAMVIR-1 at 1/100 dilution on CV-1 cells infected with 30 pfu/cell L1 vaccinia (g) or wild type vaccinia (h). Cells were fixed in 5% formaldehyde-PBS for five minutes, washed in PBS, and after incubation with CAMVIR-1 for 30 minutes at room temperature, washed again in PBS, and reacted with FITC-labelled anti-mouse IgG at 1/70 (Dako).

tein reacts with a protein of the expected molecular weight in recombinant vaccinia virus infected cells and detects the protein in a predominantly nuclear location, corresponding to earlier findings using polyclonal anti-L1-antisera.[13]

We also obtained nuclear staining of morphologically koilocytic cells on formalin fixed, paraffin wax embedded tissue sections using CAMVIR-1. It was clear from the subsequent typing of these sections using in situ hybridisation that CAMVIR-1 binds strongly to cells containing HPV-16 or -33. Very occasionally, however, a number of cells in the superficial layer of an HPV-6 containing lesion would show nuclear staining. From the results on cervical smears it is clear that cells containing very high copy numbers of HPV-11 also give positive results with CAMVIR-1. A substantial number of lesions containing HPV-6, however, were totally negative when reacted with CAMVIR-1. The antibody probably binds, to a much lesser extent, to cells containing HPV-6 or -11, so that only cells with a very high viral DNA copy number show positive staining. The epitope to which CAMVIR-1 is directed was identified as GFGAMDF (a.a.number 230–236) using overlapping peptides in an ELISA (Cason J, McCance D, personal communication). This region is highly con-

served among the different HPV types, and there is one amino acid substitution between HPV16 and HPV-6 or -11 in this region, namely, D to N (GFGAMNF), which would explain the weak cross reactivity with these virus types. CAMVIR-1 identifies an epitope on HPV-16 L1 that is strongly reactive against HPV-16 and -33 and, to a much lesser extent, with HPV-6 and -11, and thus is able to identify a subgroup of human papillomaviruses with malignant oncogenic potential.

Expression of the L1 capsid protein, as identified with this monoclonal antibody, is strongly focal, and in any one lesion only a few cells with high copy viral DNA synthesise L1 protein. This situation differs from that seen in some other lesions induced by papillomavirus, particularly cutaneous warts[19] and bovine papillomas[20] in which many cells express viral capsid proteins and in which viral particles are present in substantial numbers. The focality of L1 expression in the cervical lesions may be related to the paucity of viral particles in lesions containing HPV 16. Alternatively, it may be related to the stage of infection at which the cervical lesion has been biopsied. The natural history of HPV-16 related disease is virtually unknown. It is evident from studies on other papillomaviruses, particularly CRPV and BPV-1, that the development of a lesion in which viral capsid protein and viral particles are produced requires a considerable time period—of the order of months.[21] Anecdotal evidence in human disease suggests that a similar time period is involved in the development of genital condylomata.[22] In many patients biopsy specimens may be taken at a stage in the natural history of the disease when viral particles are not produced, either because the lesion is at an early stage or because a quiescent or persistent phase is another feature of the disease process. Whatever the explanation it is evident that L1 capsid protein is synthesised in a few cells containing high numbers of viral DNA copies in the superficial, terminally differentiated surface layers of the epithelium.

Detection of viral DNA only informs about presence of the genome and gives little information on virus replication and pathogenesis. This is particularly important in that papillomaviruses are strictly epitheliotropic, and the available information indicates that permissive viral replication only occurs in keratinocytes and specifically in the keratinocyte undergoing the normal process of differentiation in the epithelium. The intra-epithelial neoplastic lesions of the cervix are characterised by a progressive loss and distortion of the normal differentiation process, and these features are included in the diagnostic criteria of these lesions.[23] Changes in the expression of viral proteins may be a reflection of neoplastic phenotype and may give useful information on progression and prognosis, and the antibody CAMVIR-1 may be a useful reagent in this context.

We are grateful to Drs Lynn Kennedy and Ian Frazer of the Department of Medicine and Lions Immunology Laboratory, University of Queensland, Australia, for the provision of HPV typed smears; the Histopathology Department, Addenbrooke's Hospital, Cambridge, for tissue sections of cervical biopsies; and to Mrs Mary Wright for typing the manuscript.

1 zur Hausen H. Human papillomaviruses and their possible role in squamous cell carcinomas. *Curr Top Microbiol Immunol* 1977;**78**:1–30.
2 Durst M, Gissmann L, Ikenburgh H, zur Hausen H. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc Natl Acad Sci USA* 1983;**80**: 3812–5.
3 Campion MJ, McCance DJ, Cuzick J, Singer A. The progressive potential of mild cervical atypia: a prospective cytological, colposcopic and virological study. *Lancet* 1986;**ii**:237–40.
4 Schneider A, Kraus H, Schuhmann R, Gissmann L. Papillomavirus infection of the lower genital tract: detection of viral DNA in gynaecological swabs. *Int J Cancer* 1985; **35**:443–8.
5 Meanwell CA, Cox MF, Blackledge G, Maitland NJ. HPV-16 DNA in normal and malignant cervical epithelium: implications for the aetiology and behaviour of cervical neoplasia. *Lancet* 1987;**i**:703–7.
6 Wickenden C, Steele A, Malcolm ADB, Coleman DV. Screening for wart virus infection in normal and abnormal cervices by DNA hybridisation of cervical scrapes. *Lancet* 1987;**i**:65–7.
7 Young LS, Bevan IS, Johnson MA, *et al.* The polymerase chain reaction: a new epidemiological tool for investigating cervical human papillomavirus infection. *Br Med J* 1989;**298**:14–8.
8 Li CH, Shah KV, Seth A, Gilden R. Identification of the human papillomavirus type 6bL1 open reading frame protein in condylomas and corresponding antibodies in human sera. *J Virol* 1987;**61**:2684–90.
9 Matlashewski G, Banks L, Wu-Liao J, Spence P, Pim D, Crawford L. The expression of human papillomavirus type 18 E6 protein in bacteria and the production of anti-E6 antibodies. *J Gen Virol* 1986;**67**:1909–16.
10 Banks L, Spence P, Androphy E, *et al.* Identification of human papillomavirus type 18 E6 polypeptide in cells derived from human cervical carcinomas. *J Gen Virol* 1987;**68**:1351–9.
11 Doorbar J, Gallimore PH. Identification of proteins encoded by the L1 and L2 open reading frames of human papillomavirus 1a. *J Virol* 1987;**61**:2793–9.
12 Doorbar J, Campbell D, Grand RJA, Gallimore PH. Identification of the human papillomavirus 1a E4 gene product. *EMBO J* 1984;**5**:355–62.
13 Browne HM, Churcher MJ, Stanley MA, Smith GL, Minson AC. Analysis of the L1 gene product of human papillomavirus type 16 by expression in a vaccinia virus recombinant. *J Gen Virol* 1988;**69**:1263–73.
14 Koher C, Howe SC, Milstein C. Fusion between immunoglobulin-secreting and non-secreting myeloma cell lines. *Eur J Immunol* 1976;**6**:292–5.
15 McLean C, Buckmaster A, Hancock D, Buchan A, Fuller A, Minson A. Monoclonal antibodies to three non-glycosylated antigens of herpes simplex virus type 2. *J Gen Virol* 1982;**63**:297–305.
16 Gowans EJ, Burrell CJ, Jilbert AR, Marmion BP. Cytoplasmic (but not nuclear) hepatitis B virus (HBV) core antigen reflects HBV DNA synthesis at the level of the infected hepatocyte. *Intervirology* 1985;**24**:220–5.
17 Oltersdorf T, Seedorf K, Rowekamp W, Gissmann L. Identification of human papillomavirus type 16 E7 protein by monoclonal antibodies. *J Gen Virol* 1987;**68**:2933–8.
18 Patel D, Shepherd PS, Naylor JA, McCance DJ. Reactivities of polyclonal and monoclonal antibodies raised to the major capsid protein of human papillomavirus type 16. *J Gen Virol* 1989;**70**:69–77.
19 Roseto A, Pothier P, Guillemin M-C, *et al.* Monoclonal antibodies to the major capsid protein of HPV1. *J Gen Virol* 1984;**65**:1319–24.
20 Jenson AB, Rosenthal C, Olson C, Pass F, Lancaster WD, Shah K. Immunological relatedness of papilloma viruses from different species. *JNCI* 1980;**64**:495–500.
21 Olson C. Animal papillomaviruses: historical perspectives. In: Salzman NP, Howley PM, eds. *The Papovaviridae Vol. 2. The Papillomaviruses*. New York: Plenum Press, 1987:39–66.
22 Oriel DJ. The natural history of genital warts. *British Journal of Venereal Diseases* 1971;**47**:1–13.
23 Buckley CH, Butler EB, Fox H. Cervical intraepithelial neoplasia. *J Clin Pathol* 1982;**35**:1–13.