Malignant transformation of a papilloma induced by bovine papillomavirus type 4 in the nude mouse renal capsule

Jonathan Gaukroger,1 Lata Chandrachud,1 William F. H. Jarrett,1 Gail E. McGarvie,2 W. Andrew Yeudall,2 Roselyn E. McCaffery,2 Kenneth T. Smith2 and M. Saveria Campo2*

1Department of Veterinary Pathology, University of Glasgow and 2The Beatson Institute for Cancer Research, Cancer Research Campaign Beatson Laboratories, Garscube Estate, Glasgow G61 1BD, U.K.

A papillomatous cyst was induced by implanting bovine foetal palate epithelium, infected in vitro with bovine papillomavirus type 4 (BPV-4), beneath the renal capsule of a nude mouse. The benign tumour underwent malignant progression, developing into a squamous cell carcinoma with metastatic deposits in the spleen. The bovine origin of both the renal and splenic cancers was confirmed by the presence of bovine major histocompatibility complex class I antigens in the cancer cells and by sequencing the Harvey-ras 1 gene, which was shown to be of bovine origin. BPV-4 DNA was present in the residual papillomatous fronds of the renal cancer, but was absent from the carcinoma proper and for the splenic metastasis. These results confirm that BPV-4 is a carcinogenic agent and that its genetic information is not necessary for the maintenance of the malignant phenotype. Moreover the system provides the opportunity to investigate the role of viral and chemical carcinogens in an experimental system.

Bovine papillomavirus type 4 (BPV-4) is one of the causative agents of squamous cell carcinoma of the upper alimentary canal in cattle (Jarrett et al., 1978; Campo et al., 1980); other co-factors are immunosuppressants and chemical co-carcinogens present in the bracken fern (Jarrett et al., 1978; Campo & Jarrett, 1986). Although the viral aetiology of the cancer is well established, the molecular mechanisms by which the virus brings about cell transformation are not well understood because the viral DNA is absent both from frank cancers (Campo et al., 1985) and cells transformed in vitro (Smith & Campo, 1988, 1989), strongly suggesting that the virus is responsible for early events in carcinogenesis, but not for the maintenance of the transformed phenotype.

We have reported recently the induction of virus-producing papillomas by implantation of BPV-4-infected foetal palatine mucosa in the renal capsule of the athymic mouse (Gaukroger et al., 1989), using the technique pioneered by Kreider and colleagues (Kreider et al., 1986, 1990). Here we show the conversion to malignancy and metastasis of one of these papillomas.

The infection of foetal bovine palatine tissue fragments with BPV-4 and the insertion of the infected tissue beneath the renal capsule of nude mice were as described (Gaukroger et al., 1989). The mice were killed 16 weeks after implantation; half of each kidney was frozen in liquid nitrogen for DNA hybridization analysis and the other half was fixed in buffered formalin and embedded in paraffin for standard histology, immunocytochemical staining and in situ hybridization. Of the mice, 90% (19/21) had developed papillomas which produced virus, identified by the positive staining of a peroxidase-antiperoxidase (PAP) reaction, which detects the capsid antigens of BPV-4 and is diagnostic of virion production (Jarrett et al., 1984). One of the papilloma cases was accompanied by a carcinoma which had infiltrated the mouse kidney (Fig. 1a); the PAP staining of this carcinoma was confined to small areas in the papilloma fronds and was absent from the cancer (Fig. 1a, b). This appears to be similar to the absence of virus or viral antigens from the alimentary canal carcinomas of cattle (Jarrett et al., 1978). The spleen of the same animal was heavily infiltrated by metastatic deposits (Fig. 1c) in which no PAP staining was observed, pointing to the absence of viral antigens from the tissue (data not shown).

BPV-4 transforms mouse cells (Campo & Spandidos, 1983; Smith & Campo, 1988) and induces sarcomas in hamsters (Moar et al., 1986). To eliminate the possibility that the cancers had arisen by transformation of mouse cells, either spontaneously or induced by BPV-4, histological sections were incubated with an anti-bovine major histocompatibility complex (MHC) class I IgG monoclonal antibody (MAb) linked to alkaline phosphatase; mouse liver was used as a control. The sections were incubated with the MAb at room temperature for 1 h, washed with phosphate-buffered saline (PBS) and stained for 15 min with the substrate 5-bromo-4-chloro-3-indolyl phosphate nitro blue tetrazolium. The sections were washed in PBS, counterstained with light green and mounted. In the kidney, strong positive staining was
Fig. 1. Primary tumour of the kidney and metastatic deposit in the spleen. (a) PAP staining of viral antigens in the kidney tumour. P, Papillomatous fronds; C, carcinoma. (b) Higher magnification of a PAP-stained papillomatous frond. (c) Haematoxylin and eosin staining of the spleen. M, Metastatic infiltrate. (d) Bovine MHC class I staining of the kidney; the section was counter-stained with light green. P and C, as in (a); K, kidney tissue. (e) Bovine MHC class I staining of the spleen, with no counter-stain. M, Metastatic infiltrate; S, spleen tissue. (f) In situ hybridization of BPV-4 DNA in the kidney. P, C and K as in (d). The bar markers represent (a and c) 100 μm, (b) 30 μm and (d, e and f) 200 μm.
observed around the papilloma fronds and weaker staining in the cancer (Fig. 1d). Widespread pockets of staining were also seen in the spleen (Fig. 1e), but no staining was observed in the mouse kidney (Fig. 1d) or liver sections (data not shown). These results confirmed the specificity of the MAb for bovine cells and showed that both primary and metastatic tumours were of bovine origin. The weaker reactivity of the MAb with the cancer cells may be due to down-regulation of MHC antigen production, as reported in other cancer cases (Krief et al., 1989), including human papillomavirus-associated cervical carcinomas (Connor & Stern, 1990).

The nature of the cancers was confirmed by analysis of the ras gene by the polymerase chain reaction (PCR). Amplimers spanning the full length of exons 1 and 2 of bovine Harvey-ras 1 (McCaffery et al., 1989) were hybridized to total kidney DNA and subjected to PCR amplification as previously described (Campo et al., 1990). Fragments of the expected length were obtained and their nucleotide sequences were determined. Bovine and murine Harvey-ras genes are easily distinguishable owing to differences in the third codon position (McCaffery et al., 1989; Campo et al., 1990). The nucleotide sequence obtained was that of the bovine Harvey-ras gene (data not shown), thus giving independent confirmation that the tumour cells were indeed bovine. As previously reported for alimentary canal carcinomas in cattle (Campo et al., 1990), no mutations were observed in codons 11 to 13 and 60 to 61 (data not shown).

The presence and configuration of BPV-4 DNA in the tumours was analysed by Southern blot hybridization and by in situ hybridization. Total kidney DNA was digested with BamHI, EcoRI and HindIII, blotted and hybridized to a 32P-labelled BPV-4 probe (Campo et al., 1985). Viral DNA was present in multiple episomal copies and its restriction pattern was identical to that of the input virus DNA (data not shown). Southern blot analysis was not performed with the splenic cancer DNA as all the spleen had been fixed in formalin and embedded in paraffin. Both renal and splenic cancers were analysed by in situ hybridization using a BPV-4 DNA probe labelled with digoxigenin-dUTP (DIG-11-dUTP; Boehringer Mannheim) by the random primer method. Paraffin sections were de-waxed and incubated with 1 μg/ml proteinase K in 0.1 M-Tris–HCl pH 8 for 30 min at 37 °C. Sections were immersed in 0.25% acetic anhydride in 0.1 M-triethanolamine, pH 8, for 10 min to reduce background binding and prehybridized in 50% formamide, 2 × SSC for 1 to 2 h at 37 °C. Hybridization was carried out using 10 ng digoxigenin-labelled DNA in 20 μl hybridization solution (50% formamide, 2 × SSC, 10% dextran sulphate, 0.25% bovine serum albumin, 0.25% Ficoll 400, 0.25% polyvinylpyrrolidone, 250 mM-Tris–HCl pH 7.5, 250 μg denatured salmon sperm DNA per ml) for each slide and incubating for 16 to 20 h at 42 °C. The sections were then washed twice in 2 × SSC for 10 min at room temperature and in 0.1 × SSC for 30 min at 42 °C. The incubation with sheep polyclonal anti-digoxigenin Fab fragments conjugated to alkaline phosphatase and the colour reaction were performed as recommended by the manufacturers; the sections were then stained in 1% aqueous eosin and mounted in glycergel (Dako).

In the kidney, hybridization was detected in the papilloma fronds (Fig. 1f), where many more cells were positive for viral DNA than for viral antigens, suggesting that the majority of cells harbouring viral DNA were not permissive for late antigen expression. No hybridization was detected in the cancerous portion of the kidney (Fig. 1f) or in the splenic cancer (data not shown), suggesting that, within the detection limits of the assay, BPV-4 DNA was not present in cancerous tissue, in agreement with the observations made in primary and metastatic cancers in cattle (Campo et al., 1985). As in situ hybridization will only detect cells containing at least approximately 20 to 30 viral genomes per cell, the possibility that viral DNA was present in the cancers in very low amounts could not be discounted. The PCR, which allows amplification of minute amounts of nucleic acids, was therefore employed.

As the whole spleen had been embedded in paraffin, DNA was obtained by de-paraffinizing the tissue. The same procedure was used with kidney in order to process the two tissues in an identical way. In addition, kidney DNA extracted from deep-frozen material was also used. Sections of paraffin-embedded material (5 μm) were de-waxed by mixing in xylene and the pelleted material was washed twice with 70% ethanol, dried under vacuum and resuspended in sterile distilled water. The DNA was annealed with amplimers spanning 170 nucleotides of the E7 open reading frame (ORF) and 134 nucleotides of the L2/L1 region of BPV-4 (Fig. 2a). PCR was carried out for 30 cycles at 94 °C (1 min), 55 °C (30 s) and 72 °C (1 min), followed by a final annealing step at 55 °C (30 s) and extension at 72 °C (10 min). Amplified fragments of the expected length were obtained from the kidney (Fig. 2b); these were sequenced and shown to be identical to prototype BPV-4 DNA (data not shown). The same result was obtained with DNA from deep-frozen kidney (data not shown), showing no difference between the two methods. No amplification of viral DNA was ever seen with DNA from the splenic cancer (Fig. 2b). We conclude that the splenic cancer cells were devoid of BPV-4 DNA; the viral DNA detected in the kidney, both by Southern blot hybridization and by PCR, was probably present in the residual papillomatous fronds only, as suggested by the in situ hybridization results, but the contribution of a small number of cancer cells with
low amounts of viral DNA cannot be ruled out. Even if cells containing BPV-4 DNA were present in the renal cancer, the splenic metastasis had originated from cells which no longer harboured viral DNA. Therefore, in this experimental system, as in cattle, progression to and maintenance of malignancy do not require the continuous expression of viral functions.

In the nude mouse system we have so far observed transformation to cancer of only one papilloma out of a total of 57 animals in different experiments, consistent with carcinogenesis being the sum result of a series of rare events. We are currently observing large numbers of mice to establish the frequency of malignant conversion. The system promises to provide the opportunity to investigate the role of BPV-4 and other co-carcinogens in an experimental system more easily manipulated than cattle.

**Note added in proof.** PCR amplification of the Harvey-ras gene was performed on spleen cancer DNA obtained from de-paraffinized tissue sections. The nucleotide sequence of the amplified fragments was that of bovine Harvey-ras.

We are indebted to Dr Alan Teale of ILRAD, Nairobi, Kenya, for providing the monoclonal antibody to bovine MHC through Dr Liz Glass (AFRC, Roslyn, Edinburgh, U.K.). and to Dr Allan Balmain of the Beatson Institute for paraffin blocks of normal mouse liver. We also wish to thank Mr Alan Bradley for his expert technical assistance with the implantation experiments. We thank the Cancer Research Campaign for continuous support. M.S.C. is a CRC Life Fellow. W.A.Y. was the recipient of Research Training Fellowship G84/1855 from the Medical Research Council.

**References**


(Received 31 October 1990; Accepted 15 January 1991)