Association of Bovine Papillomavirus Type 2 and Bracken Fern with Bladder Cancer in Cattle


ABSTRACT

The bladder cancer syndrome that often accompanies chronic enzootic hematuria in cattle grazing on pastures infested by bracken fern has been experimentally reproduced in animals fed a diet of bracken. The experimentally induced tumors were histologically and pathologically indistinguishable from the naturally occurring ones and comprised two main types: (a) carcinoma of the urothelium identical to that seen in humans; and (b) hemangioendotheliomas of the subjacent capillaries. Often the two types of tumor occurred together in the same bladder. In animals experimentally immunosuppressed with azathioprine “bracken cancer” developed in the bladder lining. DNA of bovine papillomavirus (BPV) type 2 was found in 46% (7 of 15) of the natural cancer cases and in 69% (9 of 13) of the experimentally induced lesions, independently of histological type and including the hemangiomata of the azathioprine-treated animals, suggesting a close association between BPV and bovine bladder neoplasia. Moreover, BPV-2 DNA was found in experimental animals that had not been inoculated with BPV at all or had been inoculated with a different BPV type and had been kept in isolation, suggesting that BPV can persist in a latent state and be activated when the animal is exposed to the bracken cocarcinogens and to immunosuppressants.

INTRODUCTION

Chronic enzootic hematuria is a disease of cattle in various localized areas of the world (1), which is often associated with neoplasia of the urinary bladder (2), involving the epithelium and the mesenchyme. The disease has been associated with the presence of bracken fern in the diet of the animals, either as fresh fronds in the pasture or as dried leaves in hay (3). The plant is known to contain both mutagens and carcinogens (4) and is immunosuppressants (5). In the Scottish Highlands the high frequency of neoplasia of the upper and lower alimentary canal of cattle is epidemiologically associated with infection by BPV-4 and ingestion of bracken fern. Thirty % of the animals presenting with alimentary cancers are also affected by lesions of the urinary bladder, which include hemangiomas, hemangiosarcomas, fibromas, transitional cell carcinomas, and adenocarcinomas (6).

In August 1979 we started an experiment designed to reproduce in controlled conditions the synergism between BPV-4 and bracken as observed in naturally occurring carcinogenesis of the upper alimentary canal, and for this reason the animals were injected with BPV-4, the papillomavirus specific for the mucous epithelium of the alimentary tract (7).

MATERIALS AND METHODS

Experimental Plan. These experiments were primarily designed to reproduce in controlled conditions the synergy between BPV-4 and bracken as observed in naturally occurring carcinogenesis of the upper alimentary canal, and for this reason the animals were injected with BPV-4, the papillomavirus specific for the mucous epithelium of the alimentary tract (7).

Thirty-six young animals, ages approximately 3-5 months and born of papillomatisis-free mothers, were divided into eight groups (Table 1); group 1 (animals 1-6) was made up of six Ayrshire bull calves which were inoculated with BPV-4; group 2 (animals 7-12) was made up of five Ayrshire bull calves and one Friesian heifer; these animals were inoculated with BPV-4 and treated with the immunosuppressant azathioprine; group 3 (animals 13-16) was made up of four Friesian bull calves treated only with azathioprine; group 4 (animals 17-20) was made up of four bull calves of mixed breed and was used as control; group 5 (animals 21-26) was made up of six bull calves of mixed breed which were fed a diet of bracken fern; group 6 (animals 27-32) was made up of six bull calves of mixed breed inoculated with BPV-4 and fed with bracken; group 7 (animals 33-34) was made up of two bull calves given quercetin, the flavonoid present in bracken which has been shown to be mutagenic in both prokaryotic (9, 10) and eukaryotic (11-13) cells and reported to be carcinogenic in experimental animals (14); group 8 (animals 35-36) was made up of two bull calves inoculated with BPV-4 and given quercetin.

The groups were housed in semiisolation in separate, clean, well ventilated pens. The animals were cared for in complete accordance with the directives of the Home Office of Great Britain.

BPV-4 Inoculation. BPV-4 was isolated and purified from a single case of esophageal frond papilloma as previously described (7), and its genome was typed by restriction enzyme analysis (7). The designated animals were sedated with Rompun, 0.5-0.75 ml/kg body weight. The virus was inoculated by shallow injection beneath the epithelium at the right side of the dorsum of the tongue and the right side of the soft palate. Immediately after inoculation the pharynx was scarified in all calves by means of a bristle bottle brush.

Treatment with Azathioprine. The azathioprine solution was prepared by dissolving 2 g of azathioprine powder (Calmic Medical Division, The Wellcome Foundation, Ltd.) in 90 ml sterile saline solution by dropwise addition of 7.24 ml 1 N NaOH. In some cases a slight excess of alkali was required to ensure full dissolution of the powder. The solution was prepared up to 3 days in advance and stored at 4°C. It was administered daily to the designated calves by s.c. injection at a dose of 2 mg/kg body weight.

Bracken Fern Feeding. Fresh bracken was collected daily, and only the upper softer parts of the plant were used. Between 20 and 25 kg of bracken were divided between the two pens housing the designated animals every day from the beginning of June to the end of September, with an interval of 3 weeks after the first 6 weeks. During the winter months the animals were fed on hay. The bracken feeding cycle was repeated every year to the end of the experiment. The quantities eaten by each calf are unknown because ad libitum feeding was used.

Treatment with Quercetin. Quercetin (5,7,3'-4'-tetrahydroxyflavone; Sigma) was dissolved in dimethyl sulfoxide and ethanol and administered p.o. to the designated calves at a dose of 1 g/calf/day for 5 months. Thereafter the dose was increased to 20 g/calf/day. Treatment was suspended for a month every 4 months.

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1 Thanks are due to the Cancer Research Campaign for continuous financial support.
2 CRC Fellow. To whom requests for reprints should be addressed.
3 Present address: Quality Biotech, West of Scotland Science Park, Kelvin Campus, Garscube Estate, Glasgow, Scotland.
4 The abbreviations used are: BPV-4, bovine papillomavirus type 4; PBL, peripheral blood leukocyte(s).
Table 1 Experimental plan

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal</th>
<th>BPV-4</th>
<th>Azathioprine</th>
<th>Bracken</th>
<th>Quercetin</th>
<th>Lesion*</th>
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*He, hemangioma; HS, hemangiosarcoma; HeN, hemangioendothelioma; Ca, transitional cell carcinoma; Po, polyps; M, metastases; sa, still alive. + and - indicate presence or absence, respectively.

Collection of Blood Samples. Blood samples were collected from every animal at monthly intervals by jugular venepuncture for hematology, biochemistry, serum preparation, and PBL estimation.

Collection of Naturally Occurring Bladder Cancers. Cancers were obtained from diseased animals referred to the veterinary school by local farmers and veterinarians from bracken-infested areas. One half of each specimen was used for histopathology and the other one half was frozen in liquid N₂ as soon as possible after excision and then stored at -70°C until needed for DNA analysis.

Collection of Experimentally Induced Bladder Cancers. Cancers were obtained at autopsy and processed as above.

Collection of Normal Bladder Tissue. Normal bladder tissue was obtained from the local abattoir.

BPV Sequences in Bladder Tissue. The isolation and purification of tissue DNA, restriction enzyme analysis, and Southern blot hybridization were as described (15). The hybridization probes were DNA from recombinant BPV-1, BPV-2, and BPV-4 (16) radioactively labeled either by nick-translation (17) or by random priming (18).

RESULTS

Effect of Bracken Eating on the Immune Status. Bracken feeding caused two marked hematological changes which are associated with immunosuppression. The first of these was a dramatic fall in polymorphonuclear leukocytes. If unchecked, this leads to severe acute immunosuppression with invasion of the bloodstream by alimentary bacteria and death from septi-

cemia. This is the well described veterinary syndrome of acute bracken poisoning. Acute bracken poisoning occurred in all of our bracken-fed animals. As soon as it was detected hematologically, bracken feeding was stopped; in the following week or two the polymorphonuclear cell count rose to normal levels and bracken was restored to the diet. This was a cyclic pattern through the summer months when bracken was available (Fig. 1A). The second effect of bracken feeding is a chronic drop in circulating lymphocytes. After approximately 34 weeks from

the beginning of bracken feeding the lymphocyte count was very low and remained so throughout the experiment (Fig. 1B).

Effect of Azathioprine on the Immune Status. Administration of azathioprine produced a marked drop in PBL in all treated animals. An example is given in Fig. 1C; in this particular animal (group 2, number 9), PBL dropped from $6.7 \times 10^9$/liter to $2.2 \times 10^9$/liter over the course of 1 year. Because of the severe immunosuppression and continuous hematuria, the azathioprine treated animals were killed after 1 year of treatment.

Effect of Quercetin on the Immune Status. No effect on the immune status of the animals in groups 7 and 8 was noticed; the number of PBL remained constant and within normal limits. Bladder Cancers in Bracken-fed Animals. All animals injected with BPV-4 developed alimentary canal papillomas. The effects of bracken, azathioprine, and quercetin on the development and progression of these tumors will be described elsewhere.

The two groups of bracken-fed animals (group 5, animals 21–26, bracken diet only; and group 6, animals 27–32, bracken plus BPV-4) will be considered together because there were no differences between them regarding tumor development in the urinary bladder.

Animals 21, 22, 25, 27, and 28 were killed in November 1981 because of continuous hematuria. Visual inspection showed that the fundus of the bladder of these animals was covered in neoplastic folds with markedly thickened urothelium and several hemangiomas, ranging from 2 mm to 6 mm in diameter (Fig. 2A). Histological examination revealed that several of the hemangiomas were in fact hemangiosarcomas (Fig. 2B) or hemangioendotheliomas in animals 21, 22, 27, and 28. Animal 22 had, in addition, carcinoma of the transitional epithelium (Fig. 2C), which had metastasized to the local lymph nodes. Animal 25 had transitional cell carcinoma.

Animal 23 was killed in January 1983. It had been passing large amounts of blood during the previous week and over the last 2 days had developed urinary retention. The main lesion was a grossly distended bladder full of blood clots blocking the urethra. The whole internal surface of the bladder was covered with large hemangiomas, 20 of them greater than 1 cm in diameter; there were also carcinomas. Histologically, these were confirmed as hemangiosarcomas, hemangioendotheliomas, and transitional cell carcinomas, respectively.

Animals 32 was killed in March 1985 and Animal 30 in September 1985. In Animal 32, the wall of the urinary bladder was thickened with a mat of fronded epithelium approximately 7 mm deep, with several discrete polyps, the biggest of which was 2 cm in diameter attached by a stalk 3 cm in length and 0.5 cm thick. There were a few isolated hemangiomas measuring 3 mm in diameter. There was a nodular submucosal mass approximately 40 cm in diameter, which had extended to the left ureter and to the aorta and iliac arteries with penetration to the lumen in each case. Histological examination confirmed the presence of transitional cell carcinoma with massive infiltration of the submucosal lymph nodes. Within the carcinoma there were areas of glandular metaplasia and squamous metaplasia. There were frequent areas of collision between carcinoma and hemangiosarcoma. Animal 30 presented with similar lesions. In this case, the carcinoma had infiltrated the right media iliac, the left media iliac, and the superficial inguinal lymph nodes.

Animal 26 was killed in December 1987. Although some parts of the bladder were heavily vascularized, there was no obvious hemangiomatous tissue. However, the surface of the bladder was covered with transitional cell carcinoma. Histology showed that this ranged through all grades and stages. It had invaded the whole bladder wall penetrating the surrounding tissues and the lymphatic channels where it caused marked fibrosis and had infiltrated the lymph nodes. The ureter had a normal epithelium but its wall near the point of entry into the bladder had been heavily infiltrated by anaplastic tumor cells.

Animal 31 was killed in November 1989. The whole surface of the bladder was covered with several hundred hemangiomas ranging from 3 cm in diameter to pinpoint size. Several were bleeding and several were mixed with transitional cell carcinoma. The carcinomas ranged from one which was completely papillomatous with a very thin stalk to several which were cecal and firmly attached to the epithelium. Histology showed no evidence of penetration of the bladder wall and no lymph node involvement.

Animal 24 was killed in March 1990. The bladder had several hemangiomas largely scattered over the fundus and covering the whole area. Also spread all over the fundus were grade I transitional cell carcinomas, ranging from plaque-like lesions to protruding papilliform tumors with many intermediate cecal tumors. Histology showed no penetration of the tumors into the bladder wall.

Animal 29 died in October 1992. The bladder had hemorrhagic papillomas, transitional cell carcinoma covering the entire mucosal surface and infiltrating the bladder wall, and hemangiosarcomas.

Bladder Abnormalities in Azathioprine-treated Animals. Group 2 (animals 7–12, azathioprine plus BPV-4) and group 3 (animals 13–16, azathioprine only) will be described together. These animals were killed in September 1981 because of continuous hematuria.

In all cases there were pinpoint hemorrhagic lesions on the bladder surface, which in animal 12 extended over an area of approximately 10 x 10 cm in the distal part of the bladder. Histological examination revealed that these lesions consisted of areas of subepithelial edema with focal proliferation of capillaries. These caused small corrugations of the bladder lining, typical of the early lesions in areas surrounding “bracken type” hemangiomias (Fig. 2D). The base of some of the folds contained small lymphoid aggregates.

Urinary Bladder Status in Group 1 and 4 Animals. In marked contrast to the animals in groups 2, 3, 5, and 6, no bladder lesions were observed in these animals, which either had been infected with BPV-4 only (group 1) or had received no treatment (group 4). Histological examination revealed normal bladder lining and walls.

Animals in Groups 7 and 8. Neither group 7 (quercetin alone) nor group 8 (quercetin and BPV-4) animals showed any sign of hematuria during the course of the experiment; they were no different from the control animals in groups 4 and 1 and were not histologically examined at postmortem. The animals in group 8 were no different from animals in group 1 also with respect to p.o. papillomatisos, which was confined to the injection sites (not shown).

Bladder Cancers in Field Cases. The 15 cases of naturally occurring bladder tumors (animals A–O; Fig. 2E) comprised transitional epithelium carcinomas (Fig. 2F), papillary carcinomas (Fig. 2G), hemangiomias (Fig. 2H), hemangiosarcomas, fibromas, and polyps. Six animals had more than one type of bladder tumor (Table 2); in addition, two animals had rumen fibropapillomas containing BPV-2 DNA (19), three animals had BPV-4 papillomas at several sites in the upper alimentary
BLADDER CANCER, BPV-2, AND BRACKEN FERN

canal (7), three animals had skin warts containing either BPV-1 or BPV-2 (20), and five animals had also malignancies of the upper alimentary canal and/or the bowels (Table 2). The tongue carcinoma of animal F was the only alimentary tract cancer that contained BPV-4 DNA (15).

Viral DNA in Bladder Lesions from Bracken-fed and Azathioprine-treated Animals. Genomic DNA from bladder lesions of animals 7, 8, 12 (group 2, BPV-4 plus azathioprine), 21, 22, 23, 25, 26 (group 5, bracken diet), 27, 28, 30, 31, and 32 (group 6, BPV-4 plus bracken diet) was digested with several restriction enzymes and Southern blotted. Animals 22, 23, 28, 30, 31, and 32 had multiple cancer types (Table 1); DNA was analyzed from the hemangioendothelioma and transitional cell carcinoma of animal 22, the transitional cell carcinomas of animals 23 and 30, both cancer types of animals 28 and 31, and all cancers of animal 32, including the metastatic lymph nodes but excluding the hemangiosarcoma. Hybridization of the blots to BPV-1, BPV-2, and BPV-4 DNA probes showed that the majority of the biopsies contained multiple copies of episomal BPV-2 DNA (Fig. 3A); the exceptions were cases 23, 26, 30, and 31, which were consistently negative for all the probes. In the cases with multiple tumor types, the tumors were either all positive or all negative. No virus or viral antigens were detected in the bladder lesions (data not shown), indicating that the bladder epithelium is nonpermissive for virus replication.

Viral DNA in Natural Bladder Cancers. Genomic DNA from 15 naturally occurring bladder cancer biopsies (A-O) and from 10 histologically normal bladder epithelium biopsies was digested with several restriction enzymes and Southern blotted. The blot was hybridized to the BPV-1, BPV-2, and BPV-4 probes and this showed that seven of the cancer biopsies (D, F, G, H, J, N, and O) were positive for BPV-2 DNA (Fig. 3B; Table 2). The remaining cancer biopsies were negative. As for the experimental cancers, no virus or viral antigens were detected in the BPV-2 DNA-positive cancers. Eight of the normal bladder biopsies were negative; of the remaining two, one was positive for BPV-2 DNA and the other was positive for an unidentified papillomavirus (data not shown).

DISCUSSION

Urinary Bladder Cancer in Cattle Is Associated with Papillomavirus and Bracken Fern. The association between papillomavirus and cancer of the urinary bladder in cattle was first proposed by Olson et al. (21). These authors had shown that extracts of bovine cutaneous warts could induce cancer when injected into the urinary bladder of cows (21). Vice versa suspension of spontaneous, bracken-associated bovine bladder tumors produced warts of the skin and vagina as well as polyps and fibromas in the bladder of test calves (22). These observations strongly suggested the presence of papillomavirus in naturally occurring bladder cancers. However, when these experiments were performed, the existence of multiple types of BPV was not known (20, 23), and the identity of the virus was not confirmed. Moreover, the relationship between bladder malignancies and bracken fern, suggested by the experiments of Pamukcu et al. (2), was not further investigated; and the exact role of virus and bracken and the possible synergism between viral, chemical, and immunological factors in the genesis of bladder cancer were not clear. The results of our long-term experiments confirm that both virus and bracken fern are involved in bladder carcinogenesis and that the fern is indeed instrumental in the process.

![Fig. 3. BPV-2 DNA, in experimental and naturally occurring bladder cancers.](image)
BLADDER CANCER, BPV-2, AND BRACKEN FERN

BPV-2 DNA in Urinary Bladder Cancers. The virus implicated is most often BPV-2; its DNA has been detected in 69% of experimental cancers and in 46% of naturally occurring ones by conventional Southern blotting. In contrast, only 20% of the control animals had detectable BPV DNA in their bladder; in one case this was BPV-2 and in the other it was an unidentified papillomavirus. It is possible that a higher percentage of animals would have scored positive if the much more sensitive polymerase chain reaction technique had been used. These figures are similar to those reported for papillomavirus-associated genital cancer in humans, where approximately 80% of the cancers contain viral DNA detectable by Southern blotting but only approximately 20% of normal asymptomatic controls (24).

The high degree of association between bladder cancer and BPV-2 reported here and Olson's (21) early experiments shows that the virus in the skin warts was not genotyped.

Table 2 Naturally occurring tumors

<table>
<thead>
<tr>
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<th>Bladder*</th>
<th>Alimentary Tract</th>
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<tr>
<td>A</td>
<td>Ca + He</td>
<td>Rumen fibropapilloma</td>
<td>Warts (BPV-1/2)*</td>
</tr>
<tr>
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<td>Ca + He</td>
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* A, transitional cell carcinoma; Ca, transitional cell carcinoma; He, hemangioma; HS, hemangiosarcoma; F, fibroma; Po, polyps.

Immunosuppression Activates Latent Papillomavirus. Bladder cancer and hematuria in cattle are associated also with ingestion of bracken fern (3), and indeed animals experimentally fed bracken fern similarly developed hematuria and bladder tumors, both in our experiment and in the early ones of Price and Pamukcu (25) and Pamukcu et al. (2). However, papillomavirus presence in the tumors was not investigated by those authors and there was no report of papillomatisos affecting their animals, thus leaving open the question of virus involvement in bracken-related bladder carcinogenesis. The results presented here and in a preliminary report (8) suggest that latent papillomavirus can be transmitted from mother to calf at and after birth and during nursing; the virus then becomes activated in immunocompromised animals. The points to consider are the following: (a) the young calves were obtained from papillomatisis-free mothers and were papillomatisis-free themselves; (b) the calves were kept in isolated pens, and to minimize risk of cross-infection, care was taken by the stockmen in changing protective clothing and boots when moving from pen to pen; (c) the calves were experimentally infected with well characterized BPV-4 or with no virus at all; (d) most of the bladder lesions, whether the animal had been injected with BPV-4 or not, contained BPV-2 DNA; and finally (d) some, but not all, immunosuppressed animals developed skin warts harboring BPV-1 or BPV-2 at sites of damaged skin (8). Akin to our findings is the detection of HPV-11 DNA in bladder tumors of immunosuppressed renal transplant recipients (29, 30) and of HPV-16 DNA in the bladder carcinoma of a patient with mild immunodeficiency (31).

The bladder lesions in the azathioprine-treated animals were premalignant hemangiommas, whereas the lesions in the bracken-fed animals were frank cancers. We speculate that immunosuppression activates papillomavirus, but for full malignant progression of viral lesions the chemical mutagens of bracken are needed. It must be pointed out, however, that the azathioprine-treated animals had to be killed much earlier than the bracken-fed ones for humanitarian reasons, and it is possible that their bladder hemangiommas might have progressed to cancer had the animals lived longer.

BPV-2 DNA Is Present in Naturally Occurring Bladder Cancers. The animals presenting with naturally occurring bladder cancers were all from bracken-infested areas and immunosuppressed. Often they had other papillomavirus-related tumors, both benign and malignant. These comprised BPV-1/2 skin warts (20); fibropapillomas of the rumen, containing BPV-2 DNA (19); BPV-4 papillomas at several sites in the alimentary canal, and malignancies of the upper and lower alimentary tract, including a tongue carcinoma which was the only alimentary canal cancer containing BPV-4 DNA (15). Some of the animals therefore were additionally infected with BPV-2 at sites other than the urinary bladder, and the presence of BPV-2 DNA in the bladder malignancies might be explained as an opportunistic infection. However, not all the animals positive for BPV-2 DNA in their bladder cancers had other BPV-2 tumors, at least at the time of presentation, and this reinforces the argument that the virus is a causal factor in bladder carcinogenesis.

Approximately one-half of the natural bladder cancers, but only approximately 30% of the experimental ones, were negative for viral DNA. This may be due to lower amounts of viral DNA in the natural cancers, below the relatively low sensitivity of Southern blot hybridization, or, possibly, to the involvement of a BPV type unrelated to the types used as molecular probes.

The route of infection of the urinary bladder by BPV-2 is a matter for speculation. As it has been postulated for human papillomavirus in human bladder tumors (29-31), the virus may spread from genital sites, although in the British Isles

5 M. S. Campo et al., manuscript in preparation.
tumors and papillomas of the genital area are most often associated with BPV-1 (20).

Quercetin Alone Does Not Affect the Outcome of Papillomavirus Infection. Quercetin is reportedly one of the most potent carcinogenesis in cattle. It is conceivable that rats, being homogastric, metabolize quercetin differently from cattle and this may explain the difference between Pamukcu's results and ours. However, our quercetin-treated calves had no detectable effect. This contrasts with the earlier finding of Pamukcu et al. (14) that administration of quercetin induces intestinal and bladder cancer in experimental rats. It is conceivable that rats, being homogastric, metabolize quercetin differently from cattle and this may explain the difference between Pamukcu's results and ours. However, our quercetin-treated calves had no detectable effect. This contrasts with the earlier finding of Pamukcu et al. (14) that administration of quercetin induces intestinal and bladder cancer in experimental rats.

In summary, our observations support the view that immunosuppression activates latent papillomavirus giving rise to premalignant lesions and that the synergism between virus and environmental cocarcinogens results in cancer progression.

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REFERENCES

Association of Bovine Papillomavirus Type 2 and Bracken Fern with Bladder Cancer in Cattle
