Experimental reproduction of the papilloma–carcinoma complex of the alimentary canal in cattle

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Bovine papillomavirus type 4 (BPV-4) is the aetiological agent of epithelial papillomas of the upper alimentary canal in cattle. These benign tumours can become a focus for transformation to squamous cell carcinomas in animals feeding on bracken fern. Strong epidemiological evidence suggests that the progression to malignancy is due to the interplay between BPV-4 and mutagenic and immuno-suppressing chemicals present in the fern. The carcinomas of the upper alimentary canal are often accompanied by adenomas and adenoscarcinomas of the lower intestine and bracken-grazing animals are also heavily immuno-suppressed. To elucidate the individual roles and the concerted action of the viral and chemical factors involved in tumorigenesis and malignant conversion, we attempted to reproduce experimentally the cancer syndrome observed in the field. Florid persistent papillomatosis of the upper alimentary canal was reproduced in animals infected with BPV-4 and immunosuppressed either by a diet of bracken or by treatment with azathioprine; cancer of the upper alimentary tract or of the lower intestine developed only in animals infected with virus and fed on bracken fern. As in field cases, BPV-4 DNA was detected in papillomas but not in cancers. We conclude that immuno-suppression is necessary for persistence and spread of viral papillomas, that the fen mutants are responsible for neoplastic conversion of papilloma cells, and that continuous expression of viral functions is not required for the maintenance of the malignant state.

Introduction

In the Western Highlands of Scotland, cattle are affected by florid persistent viral papillomatosis of the upper alimentary canal (1) and are at much higher risk for the development of squamous cell carcinomas than lowland animals. The geographical distribution of cancer overlaps with that of the bracken fern, and strong circumstantial evidence has pointed to the fern as the environmental cofactor, suggesting that the progression of papillomas to carcinomas is due to the interplay between the viral agent and chemicals present in the plant (2).

The virus has been identified as bovine papillomavirus type 4 (BPV-4*), an epitheliotropic papillomavirus that infects solely the mucous epithelium of the alimentary canal (3). In healthy immunocompetent animals, BPV-4 infection is self-limiting; the papillomas are subjected to immunological control and regress ~1 year after infection (4). However, in bracken-grazing animals the papillomatosis may be florid and persistent. These long-lasting papillomas can become the focus for neoplastic conversion to squamous cell cancer (2).

Animals with carcinomas of the upper alimentary canal often present with adenomas and adenoscarcinomas of the lower intestine and with carcinomas and hemangiosarcomas of the urinary bladder (2); they are also immuno-suppressed. Epidemiologically these diseases have been defined as diverse manifestations of the combined effects of infection by papillomavirus and of grazing on bracken fern. Indeed the fern has been shown to contain, in addition to mutagens (5), also powerful immuno-suppressants (6).

Synergism with cofactors is a characteristic of papillomavirus (7) and the BPV-4 system provides an opportunity for studying the individual roles and the concerted action of the viral and chemical agents involved in malignant transformation. Thus, in August 1979 we started an experiment designed to reproduce in controlled conditions the co-carcinogenic action of virus and bracken, and to distinguish between the effects of the bracken mutagens and immuno-suppressants (8). The reactivation of latent virus and the induction of urinary bladder cancers in immuno-suppressed animals have been reported (9,10). Here we present the experimental reproduction of the papilloma–carcinoma syndrome in animals infected with BPV-4 and given a diet of bracken.

Materials and methods

Experimental plan

This has been described in detail in ref. (10). Briefly, 36 young animals, ~3–5 months old and born of papillomatosis-free mothers, were divided into eight groups. Group 1 (animals 1–6) was made up of six calves inoculated with BPV-4; group 2 (animals 7–12) was made up of six calves inoculated with BPV-4 and treated with the immunosuppressant azathioprine; group 3 (animals 13–16) was made up of four calves treated only with azathioprine; group 4 (animals 17–20) was made up of four control calves; group 5 (animals 21–26) was made up of six calves fed a diet of bracken fern; group 6 (animals 27–32) was made up of six calves inoculated with BPV-4 and fed with bracken; group 7 (animals 33 and 34) was made up of two calves administered quercetin, a mutagenic flavonoid present in bracken; group 8 (animals 35 and 36) was made up of two calves inoculated with BPV-4 and administered quercetin.

The animals were cared for in complete accordance with the directives of the Home Office of Great Britain.

BPV-4 purification, typing and infection

These techniques were as previously described (3,11,12). Briefly BPV-4 was purified from macerated tumour tissue on CsCl density gradients and typed by restriction enzyme mapping, Southern blotting and immunohistochemistry. Infection was by intramucosal injection into the palate under Rompun light general anaesthesia.

Haematological analysis

Animals were bled from the jugular vein every 5 days for the first 35 weeks and each month thereafter. Quantitative assays were by Coulter Counter and differential leucocyte percentages were based on 400 cells. Lymphocytes were separated from blood and other blood cells by Ficol Hypaque (Pharmacia).

Treatment with azathioprine

The azathioprine solution was prepared by dissolving 2 g azathioprine powder (Calnic Medical Division, The Wellcome Foundation Ltd) in 90 ml sterile saline solution by drospwise addition of 7.24 ml 1 N NaOH. It was administered daily to the designated calves by s.c. injection at a dose of 2 mg/kg body wt.

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 was divided between the two

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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 DMSO and
ethanol and administered to the designated calves at a dose of 1 g/calf/day
orally for 5 months. Thereafter the dose was increased to 20 g/calf/day.
Treatment was suspended for a month every 4 months.

Further details about the several experimental regimes can be found in
ref. (10).

Collection of alimentary canal tumours

Papillomas and cancers were obtained at autopsy; one half of each specimen
was used for histopathology and the other half was frozen in liquid nitrogen
as soon as possible after excision and then stored at -20°C until needed for
DNA analysis.

BPV sequences in tumour tissue

The isolation and purification of tissue DNA, restriction enzyme analysis and
Southern blot hybridization were as described in ref. (13). The hybridization
probes were DNA from recombinant BPV-4 or BPV-2 (14) radioactively
labelled either by nick-translation or by random priming.

Results

Immunosuppression by bracken eating and by azathioprine
treatment

The effect of bracken eating and of azathioprine treatment on
the immune system has been described in detail before (10).
Brieﬂy, animals eating bracken fern (groups 5 and 6) underwent
a rapid dramatic drop in polymorphonuclear leukocytes; if
unchecked this leads to death from septicemia. Bracken feeding
was therefore periodically stopped and during these times the
polymorphonuclear cell count returned to near normal levels.
The count of circulating lymphocytes was, however, chronically
reduced and remained so throughout the experiment.

Administration of azathioprine (groups 2 and 3) produced
a marked drop in peripheral blood leukocytes with severe
immunosuppression and hematuria. Given the degree of
immunosuppression these animals were killed after 2 years of
treatment.

In contrast to the dramatic effects caused by bracken feeding
and by treatment with azathioprine, administration of quercetin
(groups 7 and 8) had no effect on the immune status of
the animals.

Papillomas of the alimentary canal

All animals infected with BPV-4 developed typical epithelial
papillomas ~6 weeks later. In groups 1 and 8 the papillomas
were conﬁned to the injection sites, similar in this to naturally
occurring papillomas in immunocompetent animals (Figure
1A), and were rejected after 1 year. In contrast, in the
immunosuppressed animals (group 2, azathioprine, and group
6, bracken) the papillomas spread throughout the oro- and
nasopharynx, often coalescing in numerous large clumps
(Figure 1C). Similar to the massive papillomatosis encountered
in feld cases (Figure 1B), and did not undergo regression; the
azathioprine-treated animals still had papillomas at the time
of their death, and the bracken fed animals maintained their
tumours for the rest of their life: animal 29 died in October
1992 and, 13 years after inection, still had papillomas which
had spread from the mouth to the lower oesophagus and the
rumen. Therefore immunosuppression induced the spread of
papillomas and allowed their persistence well beyond their
average lifespan in immunocompetent animals. Spread and
persistence of papillomas did not take place in the animals
treated with quercetin, in accordance with their normal
immunological status.

Cancers of the upper alimentary canal and lower intestine

Although the two groups of azathioprine-treated animals
developed premalignant haemorrhagic hemangiomas in the
lining of the urinary bladder (10), no abnormalities were
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detected in their alimentary tract other than papillomas in group 2 animals. All the bracken fed animals (groups 5 and 6) developed urinary bladder malignancies (10), but only two animals from group 6 (bracken plus virus) developed cancers of the upper alimentary canal and the lower intestine. Animal 30 was killed in September 1985; this animal presented with typical papillomas in the palate, tongue and oesophagus (Figure 2A), two foci of carcinoma in the oesophagus, infiltrating the subjacent tissue (Figure 2B) and multiple polyps, adenomas and adenocarcinomas in the duodenum, jejunum and colon (Figure 2C and D). Animal 31 was killed in November 1989; this animal had papillomas and carcinoma of the oesophagus, similar to those described for animal 30, and adenocarcinoma of the jejunum.

No malignancies of the alimentary tract were detected in animals of the other groups.

**BPV-4 DNA in alimentary papillomas and carcinomas**

Papillomas excised 3 months after infection contained multiple copies of episomal BPV-4 DNA (Figure 3A). In contrast, and as previously reported for naturally occurring cases (4,13), the long-lived papillomas, the oesophageal carcinomas and the tumours of the lower intestine of animals 30 and 31 were...
devoid of viral DNA (Figure 3B and data not shown). The hybridization conditions were such that one copy of viral DNA per cell was easily detected in control samples (Figure 3B). Despite the presence of BPV-2 DNA in the urinary bladder cancers of these animals, no BPV-2 DNA was present in the alimentary cancers (data not shown).

Discussion

We have experimentally reproduced the alimentary canal papilloma–carcinoma syndrome, as encountered in the field, by infecting cattle with papillomavirus and feeding them on a diet of bracken, thereby confirming the viral-environmental nature of the disease. The importance of bracken-induced immunosuppression in reactivation of latent virus and in carcinogenesis of the urinary bladder in conjunction with BPV-4 has already been documented and discussed (9, 10). Here we show that the effects of combined exposure to the immunosuppressants and co-carcinogens of the fern and to BPV-4 infection result in the induction of widespread alimentary papillomatosis and of cancers of both the upper and lower alimentary tract.

We have dissociated the effects of immunosuppression from the possible effects of exposure to co-carcinogens. Immunosuppressed animals, independently from the route of immunosuppression, are not capable of containing BPV-4 infection and papillomas thus spread and persist. However, immunosuppression appears to be necessary but not sufficient for neoplastic progression, and additional carcinogenic compounds present in the fern seem to be required. This conclusion stems from the observation that azathioprine-treated animals did not develop alimentary tract malignancies, while bracken-fed animals did. A similar situation was observed in the urinary bladder: frank cancers developed only in bracken-fed animals in contrast to premalignant lesions seen in azathioprine-treated animals (10). It has to be pointed out, however, that the azathioprine-treated animals had to be killed earlier than the bracken-fed ones, and it is a formal possibility that they might have developed cancer had they lived longer.

One of the most potent mutagens present in bracken fern is the flavonoid quercetin (5,15-19). We have recently reported that quercetin rapidly synergizes with BPV-4 in the induction of fully oncogenic transformation of primary bovine fetal cells in vitro (20). However, quercetin-treated animals were no different from control animals, and did not develop any tumour other than BPV-4 papillomas. It is possible that the absence of discernible effects of quercetin treatment was due to the immunological competence of these animals, and that any transformed cell was eliminated by immunosurveillance mechanisms. This would confirm that both immunosuppression and exposure to co-carcinogens are necessary for neoplastic conversion.

In contrast to the presence of BPV-2 DNA in the premalignant and malignant lesions of the urinary bladder in the azathioprine-treated and bracken-fed animals respectively (10), BPV-4 DNA was present only in the early papillomas of the alimentary tract but not in advanced papillomas, oesophageal carcinomas or adenomas and adenocarcinomas of the lower intestine. This is the situation encountered in the corresponding naturally occurring tumours where the absence of viral genetic information is well documented (13), thus showing concordance between field cases and experimentally induced tumours also in this respect. Several possibilities can be invoked to explain this apparent paradox. The most minimalistic explanation is that there is no relation between viral papillomas and carcinomas: these would arise de novo or from papillomas with no viral etiology, and would be due solely to the carcinogenic action of bracken. Although formally possible, we do not favour this hypothesis. In addition to the arguments that have been presented before (13), the absence of alimentary papillomas and cancers in bracken-fed animals not infected with BPV-4 makes this alternative implausible. The presence of a different BPV type with a causative role in alimentary tract carcinogenesis is unlikely as well characterized BPV-4 was used in the present experiments and different molecular probes representing the spectrum of BPV types have repeatedly failed in detecting viral sequences in cancers. We have recently reported the spontaneous malignant transformation, with metastasis to the spleen, of a bovine papilloma induced by BPV-4 (21) in the nude mouse xenograft system (22). The derivation of the cancer from the viral papilloma could not be disputed, but nevertheless no viral DNA could be detected by the highly sensitive polymerase chain reaction in the malignant tissue. For all these reasons and because of results obtained in vitro (23), the most coherent explanation for the absence of viral genetic information in alimentary cancers is that the role of BPV-4 in carcinogenesis is confined to the early transformation stages. Cell proliferation induced by BPV-4 would provide a largely expanded target for the bracken co-carcinogens. The further expansion of initiated/transformed cells and protracted persistence of papillomas, both allowed by poor immunosurveillance, would increase the probability of additional events, such as activation of the ras gene (24) and amplification of the receptors for epidermal growth factor (25) with the eventual emergence of a fully malignant clone. Viral functions would no longer be needed and there would be no selective pressure for retaining the viral DNA.

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