Assay of Fractions of Bovine Urine for Carcinogenic Activity after Feeding Bracken Fern (*Pteris aquilina*)

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Summary

Urine from cattle fed fresh and dried bracken fern for more than 2 years and urine from control cattle was extracted with ethyl acetate and divided into nonacidic and acidic fractions. The residue obtained after removal of the solvent was mixed with 4 times its weight of cholesterol, and the mixture was converted to pellets with the aid of a pellet press. Pellets containing these residues were surgically implanted into the bladders of mice, and one group of mice received pure cholesterol pellets. Five of 15 mice exposed to the acidic fraction and 1 of 13 mice exposed to the nonacidic fraction of the urine of cattle fed bracken fern developed carcinomas. One of 13 mice exposed to the acidic fraction and none of 11 mice exposed to the nonacidic fraction of urine of cattle not fed bracken fern developed carcinomas. When these results were analyzed by the exact method of 2 × 2 tables, the only group of animals with a tumor incidence which was significantly different from the control mice exposed to pure cholesterol (0 carcinomas in 11 animals) were the mice exposed to the acidic fraction of the urine of cattle fed bracken fern (P < 0.05). These preliminary results suggest that 1 or more carcinogenic substances occur in the urine of cattle ingesting bracken fern.

Introduction

Tumors of the bovine urinary bladder were once common in the Pacific Northwest of North America, but now are rarely seen in the United States (7). Bladder tumors are frequently found in cattle in various parts of the world such as in Turkey, Yugoslavia, Bulgaria, Panama, and Brazil (13). These tumors are commonly associated with a syndrome known as chronic enzootic hematuria (22, 23). The geographic distribution and the endemic nature of this disease reminds one of the environmental aspect of industrial cancers of the bladder that occur in workers exposed to various chemicals. The pathologic structure and clinical course of many spontaneous bladder tumors in cattle (24) do not differ significantly from the industrial bladder tumors seen in dye workers or from the spontaneous bladder tumors observed in other individuals (19).

The etiology of the naturally occurring bovine urinary bladder tumors remains obscure. Recently, a papilloma-like virus was recovered from tumors of the bovine bladder (21). The infective agent resembles the bovine cutaneous papilloma virus in its behavior in test calves. It may be a passenger virus and not etiologically related to the bladder tumors. The bladder tumors produced with this agent (21) and with the bovine cutaneous papilloma virus (8) were self-limiting.

Bracken fern (*Pteris aquilina*) has been associated with the occurrence of chronic enzootic hematuria (13, 24, 25, 27). A high level of bracken in the diet of cattle causes a hemorrhagic syndrome. Acute bracken fern poisoning in cattle has been known for many years. The toxic factor causing the disease is thought to be cumulative. Fatal poisoning of cattle with bracken fern has been produced by many workers by feeding either the fresh fronds, sun-dried bracken, or powdered rhizomes mixed with an otherwise adequate diet (14, 25, 27), and with a fraction extracted from the bracken plant with boiling ethanol (15). Administering thiaminase, after it had been separated from the small molecular components of the bracken plant, had no deleterious effect on the bovine animal (14). These results eliminated thiaminase from consideration as the causative agent in bracken poisoning in cattle, and it has been suggested that the toxicity may be due to an unidentified substance called the "aplastic anemia factor" (14, 15).

Recently it has been shown that a low level of bracken in the ration over a period of months may produce bladder lesions (27) and tumors in cattle (25). An extract of urine from cows fed hay from a chronic enzootic hematuria region induced hemangiomatous growths in a dog and cutaneous papillomas in 2 of 20 mice (16). These results suggest that bracken fern may be one of the factors responsible for the high incidence of bladder tumors in bovine species in those parts of the world where cattle receive a continuous low level of bracken by eating the fresh plant and the dried plant which may be mixed with hay. Therefore, it was decided to test fractions of urine from cows fed bracken as well as from control cows for carcinogenic activity on the bladder epithelium of the mouse. The results of this preliminary experiment are the subject of this report.

Materials and Methods

Two Holstein heifers were fed bracken fern (*Pteris aquilina*) obtained from the vicinity of Tomahawk, Wisconsin. The syn-
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drome of acute bracken poisoning occurs in this vicinity, but
chronic enzootic hematuria has not been diagnosed. Each animal
received a daily bracken hay supplement of approximately 6
gm/kg of body weight, or according to the season of the year, a
daily supplement of fresh green bracken of approximately 8
gm/kg of body weight. The test animals and 2 controls had
the same basic diet of timothy and alfalfa hay and a standard dairy
feed supplement (1% salt, 27% corn, 27% bran, 27% oats and 18%
linseed oil meal). The calculated amount of chopped bracken
was given individually to each test animal once a day. The feeding
of bracken continued for more than 2 years. These animals were
initially used for Dobereiner et al. in a chromatographic study of
urine (13). In the present study urine samples from these animals
were collected in polyethylene bottles containing 1 ml of toluene,
and the samples were kept frozen until they could be extracted.

PREPARATION OF FRACTIONS. The chilled urine samples (3 liters
each) from bracken-fed and control cows were acidified to pH 2 by
the addition of concentrated HCl and saturated with sodium
chloride (approximately 22 gm/100 ml). They were then ex-
tracted with 3 successive half volume portions of ethyl acetate.
The 1st extraction was sometimes made difficult by the forma-
tion of an emulsion; this was broken by separating as much as
possible of the clear portions of the aqueous and organic layers
and centrifuging the residual emulsion. The 2nd and 3rd extrac-
tions with ethyl acetate usually proceeded smoothly. The com-
bined ethyl acetate solutions were dried with anhydrous sodium
sulfate and evaporated to about one-third of the original volume
under reduced pressure and at low temperature (38°C). The
ethyl acetate solution was extracted by prolonged and thorough
shaking with 15-ml portions of 20% ammonium carbonate
solution until the pH of the aqueous extract reached 8. The
organic layer was dried with anhydrous sodium sulfate and the
solvent was removed in vacuo at low temperature to obtain a
brown oily residue with an unpleasant odor (acidic fraction).

PREPARATION OF PELLETS. Each of the fractions (nonacidic and
acidic) was mixed separately with 4 times its weight of cholesterol
(purified by recrystallization from ethyl alcohol just prior to use,
with m.p. 151°C) by grinding thoroughly in a mortar. The mixture
was compressed into spheroidal pellets, 0.125 inch in diameter and
weighing 24–28 mg, with the aid of a deep rounded cup die in a
Eureka model pellet press (F. J. Stokes Corp., Philadelphia,
Pennsylvania). The dies were dusted frequently with fine
magnesium stearate powder to prevent fracturing of the pellet.
Pellets of comparable size were also prepared from purified
cholesterol.

ANIMAL SELECTION AND CARE. Swiss albino female mice (ob-
tained from Rolfsmeyer Co., Madison, Wisconsin) were housed in
screen-bottom metal cages with 5 animals to a cage. The mice
were 70–85 days old at the time of surgery and were fed a
Rockland pellet diet (purchased from A. E. Staley Mfg. Co.,
Decatur, Illinois) and water ad libitum.

CARCINOGENICITY STUDIES. Cholesterol pellets were implanted
into the bladders of groups of 25 mice by the surgical technic of
Jull (17), as modified by Allen et al. (1). Only those animals
surviving more than 180 days were evaluated for the presence of
bladder carcinomas in order that the results obtained could be
compared with the incidence of lesions reported by others (1, 3, 4,
9–12). The bladders were distended postmortem with Bouin’s
fixative injected into the urethra. The gross and microscopic
evaluation of the lesions was made using the criteria of Bonser
and Jull (5). Carcinomas which were not observed to invade the
muscular coat of the bladder were classified as Grade I; those
which were seen to invade muscle as Grade II. The incidence of
carcinomas was used to assess carcinogenicity. Probabilities of
statistical significance were evaluated by the exact method for
2 x 2 tables as done in previous studies in these laboratories (11)

Results

The acidic fraction of the urine from the bracken-fed cows
produced more experimental transitional cell carcinomas (5/15,
33%) than did the acidic fraction of urine from the control
animals (1/13, 7%) (Table 1). No bladder carcinomas were
observed in the groups of animals which were implanted with the
nonacidic fraction of urine of control cows or cholesterol pellets

<p>| TABLE 1 | INCIDENCE OF CHANGES IN MOUSE BLADDERS AND SURVIVAL OF MICE FOLLOWING IMPLANTATION OF FRACTIONS OF BRACKEN-FED AND CONTROL COW URINE IN THE BLADDER |
|-----------------------------------------------|</p>
<table>
<thead>
<tr>
<th>FRACTIONS</th>
<th>NO. OF MICE DIED OR KILLED (DAYS 180-331)</th>
<th>AV. SURVIVAL (DAYS)</th>
<th>SQUAMOUS METAPLASIA</th>
<th>PAPILOMA</th>
<th>CARCINOMAS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Grade I</td>
<td>Grade II</td>
<td>Total</td>
</tr>
<tr>
<td>Cholesterol alone</td>
<td>11</td>
<td>314</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>CCU nonacidic fraction</td>
<td>11</td>
<td>300</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>CCU acidic fraction</td>
<td>13</td>
<td>293</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>BFCU nonacidic fraction</td>
<td>11</td>
<td>307</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>BFCU acidic fraction</td>
<td>15</td>
<td>310</td>
<td>6</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

* CCU, control cow urine; BFCU, bracken-fed cow urine.

* These values were obtained by the exact method for 2 × 2 tables and indicate the probability that the tumor incidence in any group differed significantly from the incidence in the control mice (which were exposed to pure cholesterol).
alone. Since most of the bladders of the mice were inspected after the mice survived a definite period of time, no information was gained to determine the average time at which carcinomas appeared. However, 1 animal exposed to the acidic fraction of urine from bracken-fed cows died with a carcinoma 197 days following surgery. The incidence of papillomas was essentially similar in all groups of mice (Table 1).

The histologic changes in the mouse bladders showed great variations, including hyperplastic, metaplastic, and neoplastic changes. The histology of lesions (Figs. 1–11) was similar to that described by others (2, 5, 26). A relatively high incidence of tumors and a high degree of malignancy followed the implantation of the cholesterol pellets containing the acidic fraction of urine from bracken-fed cows. Two out of 5 carcinomas (Grade II) penetrated the muscle wall (Figs. 7–11), whereas the other 3 carcinomas (Grade I) were confined to the submucosa (Fig. 6). No indication of metastasis was found during the 47 weeks of the experiment.

Discussion

The hypothesis that bracken fern may be related to bovine urinary bladder tumors found its 1st experimental support in relatively long feeding trials by Rosenberger and Heeschen (27) and Pamukcu (25). Dobereiner et al. (13) found no influence on the urinary content of diazotizable aromatic amines or phenols (i.e., tryptophan metabolites or compounds capable of coupling with diazotized sulfanilic acid) as the result of feeding bracken fern. They concluded that paper chromatographic analyses may not have been suitable for the detection of differences regarding the excretion of urinary metabolites by bracken-fed and control animals or that the quality or amount of bracken fern fed to the animals may not have been adequate. By biologic test the present studies demonstrated that the acidic fraction of urine from bracken-fed cows contained 1 or more substances which induced a carcinogenic effect. Two out of 5 carcinomas (Grade II) penetrated the muscle wall (Figs. 7–11), whereas the other 3 carcinomas (Grade I) were confined to the submucosa (Fig. 6). No indication of metastasis was found during the 47 weeks of the experiment.

The nature of the carcinogenic metabolites has not been elucidated. A variety of chemicals such as astragalin, isouceritrin, rutin (20), catechol-tannins, pteraquilen, sugar, starch, aliphatic nondrying oil, and much pectose mucin (18) have been identified in bracken fern. There is no indication that the metabolites of any of these chemicals may be bladder carcinogens.

The results obtained in this study are not conclusive, but they are in agreement with the findings of others (16, 25, 27). The results suggest that this approach may be a suitable one for the identification of the active constituent in the urine of bovine species fed bracken fern.

Acknowledgments

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References


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Fig. 1. Normal bladder wall. The transitional epithelium 2-3 cells thick. × 350.
Fig. 2. Squamous metaplasia of the bladder epithelium (pellet containing acidic fraction of urine from a cow fed bracken fern). × 350.
Fig. 3. Bladder showing solid ingrowths of epithelium into subepithelial tissue (von Brunn's nest) and chronic inflammatory infiltration of the bladder wall (pellet containing acidic fraction of urine from a control cow). × 125.
Fig. 4. Moderate hyperplasia of transitional epithelium with some cells showing hydropic degeneration. Branched crypts lined with transitional epithelium (cystitis glandularis). The distinction between mere proliferation and benign neoplasia is difficult with this type of lesion (pellet containing nonacidic fraction of urine from a cow fed bracken fern). × 125.

Fig. 5. Marked hyperplasia and papillomatous growth of transitional epithelium and deep seated branched crypts lined with transitional epithelium (pure cholesterol pellet). × 125.
Fig. 6. Grade I transitional-cell carcinoma (pellet containing acidic fraction of urine from a cow fed bracken fern). × 250.

Fig. 7. Grade II transitional-cell carcinoma on the right. Transitional cell hyperplasia, ingrowths, and branched crypts lined with transitional epithelium on the left. The muscle coat is infiltrated by lymphocytes (pellet containing nonacidic fraction of urine from a cow fed bracken fern). × 20.
Fig. 8. From the same section as Fig. 7. Transitional cell carcinoma infiltrated by inflammatory cells. × 250.

Fig. 9. Grade II transitional cell carcinoma (pellet containing acidic fraction of urine from a cow fed bracken fern). × 21.
Fig. 10. From the same section as Fig. 9. Note infiltration of the cells of transitional cell carcinoma into the bladder wall. X 120.

Fig. 11. From same section as Fig. 9. Note intact transitional epithelium. Slight epithelial hyperplasia. Subepithelial tissue is infiltrated with chronic inflammatory cells. Transitional cell carcinoma. X 250.
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