Assay of Fractions of Bracken Fern (Pteris aquilina) for Carcinogenic Activity

A. M. Pamukcu, J. M. Price, and George T. Bryan

Department of Pathological Anatomy, College of Veterinary Medicine, University of Ankara, Ankara, Turkey [A. M. P.]; Scientific Divisions, Abbott Laboratories, North Chicago, Illinois 60064 [J. M. P.]; and The Division of Clinical Oncology, University of Wisconsin Medical School, Madison, Wisconsin 53706 [G. T. B.]

SUMMARY

Fresh bracken fern (Pteris aquilina) was extracted with cold and hot methanol followed by ethyl ether. Six fractions designated as G, E, F, K, H, and M were obtained. 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 bladder lumens of Swiss albino female mice, and a control group of mice received only pure cholesterol pellets. A 53% and 56% incidence, respectively, of bladder carcinomas was observed in the mice of Groups G and H. This incidence was statistically greater than that seen in the control group. A lower incidence of urinary bladder carcinomas was obtained in the mice of the other groups. These results suggest that one or more carcinogenic substances occur in bracken fern itself.

INTRODUCTION

Studies have clearly demonstrated that bracken fern (Pteris aquilina) contains carcinogenic substance(s). A low level of bracken fern fed in the daily ration produced urinary bladder cancer in cattle (19, 29, 32). Bracken fern also produced tumors at different sites in the body and demonstrated a wide range of action in different species. Recently, it was reported that bracken fern induced intestinal adenocarcinoma in rats (13, 31, 32) and in Japanese quail (Coturnix coturnix japonica) (35), pulmonary adenomas in mice, and bladder tumors in guinea pigs (12, 14, 35). Bracken fern can produce tumors in different sites of the body of the same species of animal. Pamukcu and Price (31) and Yalciner (36) were able to produce a high percentage of simultaneously occurring intestinal and urinary bladder carcinomas in rats by feeding bracken fern.

Biological tests (16–18, 30) also demonstrated that urine fractions of bracken fern-fed cattle and of cows bearing spontaneous urinary bladder tumors contained some carcinogenic substance(s) which induced tumors in the bladder of the calf, dog, rat, or mouse and tumors in the skin of mice. It is not known whether this carcinogenic activity is attributable to bracken fern itself or to its metabolites. It has been shown (22) that the carcinogenicity of the crude cycad material and of cycasin in rats depends on an intestinal bacterial flora capable of providing the enzyme β-glucosidase, necessary for the liberation of the aglycone, methylazoxymethanol. In germ-free rats, cycasin per se, whether present in the intestinal tract or absorbed and excreted, is innocuous. It was desirable to determine whether a similar mechanism operates in the carcinogenicity of crude bracken fern, or whether the fresh bracken fern itself contains the carcinogenic substance before digestion in the animal body. Therefore, it was decided to test fractions of bracken fern for carcinogenic activity on the bladder epithelium of the mouse. The results of these experiments are the subject of this report.

MATERIALS AND METHODS

Preparation of Fractions. Fresh bracken fern obtained from the vicinity of Bolu, Turkey, was extracted (Chart 1) with an excess of cold methanol until the green color of the bracken fern became light brown. The combined methanolic extractions were evaporated under reduced pressure at low temperature (36°), and the resulting dark green residue was designated as Fraction G. The remaining bracken fern was continuously extracted further with hot methanol. After this methanolic fraction was cooled overnight, a white solid material was obtained. This material was filtered and fractionated with ether into ether-soluble (Fraction F) and ether-insoluble (Fraction E) fractions. The ether-insoluble fraction could be crystallized with acetic acid, and showed a melting point of the resulting crystals at 75°. The soluble methanol fraction was evaporated to a reduced volume and cooled overnight. A solid material was obtained and filtered from the methanolic solution. This solid fraction was called Fraction K. The remaining methanol phase was further evaporated under reduced pressure and at low temperature (36°), resulting in a residue designated as Fraction H. The remaining bracken fern residue obtained after both cold and hot methanol extractions was dried and subjected to a continuous extraction with hot water. The water-soluble

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Bracken Fern Carcinogenicity

100 grams of chopped fresh bracken fern
Extracted with cold methanol with continuous stirring at room temperature until the color of bracken turned to light brown, then filtered

Bracken fern
Continuously extracted with hot methanol

Bracken fern
Continuously extracted with hot water and filtered

Fraction G
Combined methanolic extracts evaporated under reduced pressure at low temperature (36°C)

Fraction H
Filtrate evaporated under reduced pressure at 36°C

Fraction M
Filtrate reduced to 1/3 volume under reduced pressure and filtered

Solid extracted with ethyl ether
Ether-insoluble white material, crystallized from acetic acid, m.p. 75°C (Fraction E)

Ether-soluble portion (Fraction F)

Solid (Fraction K)

Filtrate evaporated under reduced pressure

Residue discarded

Chart 1. Flow diagram illustrating preparation of fractions tested for carcinogenic activity.

portion was dried under reduced pressure, and the residue was designated as Fraction M.

Preparation of Pellets. Each of Fractions G, E, F, K, H, and M was mixed with 4 times its weight of cholesterol (purified by recrystallization from ethyl alcohol just prior to use, m.p. 151°C) by grinding thoroughly in a mortar. The mixture was compressed into spheroidal pellets, 0.125 inch in diameter and weighing 22 to 26 mg, using a deep rounded-cup die in a Eureka model pellet press (F. J. Stokes Corp., Philadelphia, Pa.). The dies were dusted frequently with fine magnesium stearate powder to prevent fracturing of the pellet. Pellets of comparable mass were also prepared from purified cholesterol.

Animal Selection and Care. Swiss albino female mice (obtained from the Institute of Bacteriology, Elazig, Turkey) were housed in screen-bottomed metal cages with 5 animals/cage. The mice were 70 to 85 days old at the time of surgery, and were fed a pellet diet (Yem Sanayi, Ankara, Turkey) and water ad libitum.

Carcinogenicity Studies. Pellets were surgically implanted into the urinary bladders of groups of 40 mice by the pellet implantation technique of Jull (20) as modified by Allen et al. (1) and as used in previous studies from these laboratories (6–11, 30). One group of 40 mice served as a negative control group and was implanted with pellets of pure cholesterol. After 52 to 55 weeks the surviving mice were sacrificed. The bladders were distended postmortem with Bouin’s fixative injected into the urethra. Gross and microscopic evaluation of the lesions was made according to the criteria of Bonser and Jull (5) and Roe (33). Carcinomas which were not observed to invade the muscular layer of the bladder were classified as Grade I and those which invaded the muscle were classified 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 used previously (6–11, 30).

RESULTS

The number of animals subjected to the surgical implantation of pellets, the total number of animals surviving 365 days or more following surgery, and the incidence of microscopic changes observed in the mouse bladders are shown in Table 1. The number of mice that lived beyond 1 year varied from 28 to 50%. About 30% of the animals died within 30 days after surgery from impaction of the pellets into the urethra or from the toxicity of the fractions, especially Fractions G and F. Since the bladders of the mice were inspected after the mice had survived more than 1 year after surgery, no data were collected to determine the earliest time or the average time at which carcinomas were noted. The incidence of carcinomas observed ranged from 9% for Fraction F to 56% for Fraction H.

The incidence of benign papillomas was essentially similar in all groups of mice (Table 1). An examination of these data strongly suggests that the carcinogenic substance(s) is soluble in methanol, since a statistically significantly high incidence of bladder carcinomas was observed in Groups G (53%) and H (56%). A lower incidence of urinary bladder carcinomas was obtained following other extraction procedures. This evidence indicates that bracken fern itself contains active carcinogenic chemicals.

The histological changes in the mouse bladders showed great variation, including hyperplastic, metaplastic, and neoplastic changes. The histology of these lesions was similar to that...
studies and others (12—14, 31, 35, 36) have established significant factor in the etiology of urinary bladder cancer in No indication of metastasis was found during the 55 weeks of the experiment.

DISCUSSION

It appears that chronic ingestion of bracken fern is a significant factor in the etiology of urinary bladder cancer in the cattle of Turkey (19, 25—29, 32). The results of these studies and others (12—14, 31, 35, 36) have established beyond reasonable doubt that bracken fern is carcinogenic for the urinary bladder of cattle, rats, and guinea pigs; carcinogenic for the intestinal mucosa of rats and Japanese quail; and carcinogenic for the lungs of mice. It was demonstrated (30) that a metabolite(s) present in the urine fraction of bracken-fed cows is also carcinogenic for the urinary bladder epithelium of mice when implanted into the urinary bladder lumen. In the present experiments, it appears that the carcinogen(s) present in bracken fern need not be metabolized by a susceptible species to display its carcinogenic activity. The results of these experiments (Table 1) strongly suggest that the carcinogenic substance is soluble in methanol, for a significantly high incidence of bladder carcinomas was observed in Groups G (53%) and H (56%). A lower incidence of urinary bladder carcinomas was obtained following other extraction procedures. This evidence suggests that bracken fern itself contains active carcinogenic chemicals. However, these results do not eliminate the possibility that the metabolism by gastrointestinal flora or by the experimental animal itself is necessary for expression of the carcinogenic activity by bracken fern.

The nature of the carcinogenic substance(s) has not been elucidated. A variety of chemicals such as astragalin, isoquercitrin, rutin (24), catecholtannins, pteraquilin, sugar, starch, aliphatic nondrying oil, and much pectose mucin (21) have been identified in bracken fern. There is no indication that these chemicals or their metabolites are bladder carcinogens.

As bracken fern is used as a human food in greens or salads in the United States, New Zealand, and especially Japan (2, 3, 15, 23, 34), the high incidence of stomach cancer could be in part the result of eating bracken. Thus, it seems urgent that the carcinogenic compound(s) present in bracken fern be isolated and chemically characterized. Our investigations in this direction are in progress.

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