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Dietary Lignin, an Insoluble Fiber, Enhanced Uterine Cancer but Did Not Influence Mammary Cancer Induced by *N*-Methyl-*N*-Nitrosourea in Rats

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Abstract: *Previous investigations suggested potential breast cancer-preventive properties of dietary fiber from cabbage. The purpose of the present investigation was to determine whether lignin, a component of cabbage fiber, would protect against mammary carcinogenesis by* N*-methyl-*N*-nitrosourea (MNU) in Sprague-Dawley rats. A six-week study was conducted using diets containing 0.5–5% dietary wood lignin (a readily available, purified source). These diets were well tolerated by the rats, and a carcinogenesis study using 5 mg MNU/100 g body wt iv at 50 days of age was conducted, with the 2.5% lignin diet fed from 6 through 8 weeks of age followed by 5% lignin diet until 20 weeks after MNU. Dietary lignin and MNU treatment increased food consumption ($p < 0.05$), and body weight was slightly reduced at 10 and 20 weeks after MNU in the MNU-5% lignin diet group ($p < 0.05$). Serum estradiol was not altered by dietary lignin or MNU treatment, but uterine weights were highest in the MNU-control diet group 4 and 12 weeks after MNU. Expression of creatine kinase B, an estrogen-responsive gene, was lower in the uteri of the MNU-lignin diet group than in other groups at 20 weeks. Mammary carcinogenesis was not altered by dietary lignin. However, uterine endometrial adenocarcinoma was observed only in the MNU-lignin diet group (4 carcinomas/40 effective rats) ($p < 0.05$).*

Introduction

Human and experimental animal studies have suggested that fruit and vegetable consumption may protect against breast cancer. However, it is not clear which components or combinations of components of fruits and vegetables are protective. Experimental studies explored the relationship between dietary fiber and mammary carcinogenesis in rats. In the course of searching for new chemopreventive agents from cruciferous vegetables, our previous research probed the influence of dried cabbage and cabbage fiber on mammary carcinogenesis induced by *N*-methyl-*N*-nitrosourea (MNU) (1). Initially, studies determined that dried cabbage or collards inhibited mammary carcinogenesis when given in a control diet but not when given in a high-fat diet. For a subsequent study, the diets contained 5% dried cabbage or the residue remaining after exhaustive extraction of this amount of cabbage with organic solvents and water (3.2% residue). This study demonstrated a 33% inhibition of MNU-induced mammary carcinogenesis in female Sprague-Dawley rats fed the cabbage or the cabbage residue diet. There was no difference between the 5% dried cabbage and the 3.2% cabbage residue diets. This result suggests that the inhibition of mammary carcinogenesis by cabbage was possibly due to the fiber (the residue would be expected to consist of lignin, cellulose, and other noncellulose fibers) in the dried cabbage. Further evidence that dietary fiber can inhibit mammary carcinogenesis was reported by Cohen and co-workers (2). Their study assessed the interaction between dietary fat level and a 10% soft white wheat fiber. The wheat fiber significantly inhibited mammary carcinogenesis in F344 rats fed the high-fat diet but not in rats fed the control dietary fat diet (2). Wheat fiber is also a source of dietary lignin. Research reported here assessed the impact of wood lignin in the prevention of MNU-induced mammary cancer in rats. The hypothesis being tested was that lignin, a polymer, would be hydrolyzed to lignans, which were previously suggested to be effective in the inhibition of breast carcinogenesis because of their estrogenic (agonist/antagonist) activities. Wood lignin was used in the present experiment as a readily available purified source. We assessed the impact of lignin and the carcinogenesis protocol on circulating estradiol and uterine expression of the estrogen-responsive gene creatine kinase B because of the considerable evidence suggesting that estrogens promote cancers in estrogen-responsive tissues such as the breast and the uterus (3,4). In addition, we observed uterine adenocarcinoma and cervical squamous cell carcinoma in rats fed lignin and treated with MNU.

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Materials and Methods

Chemicals

MNU was purchased from Sigma Chemical, and its purity was checked by assessing its absorption spectrum at 390 nm.

Experimental Diets

The control and lignin-supplemented diets are shown in Table 1. The 5% lignin diet was formulated by adding lignin at the rate of 5 parts/100 parts control diet. The control diet was formulated in accordance with the recommendations of the American Institute of Nutrition (5,6). Our previous studies with cabbage and cabbage residue diets (1) revealed that the rats ate enough of the supplemented diets to compensate for the additional fiber (105% of the control intake). This allowed control and experimental animals to consume the same amounts of vitamins, minerals, protein, and cellulose fiber per day. Intermediate lignin supplementation (0.5%, 1%, and 2.5%) was conducted using the same approach. Lignin AT was obtained from Westvaco Polychemicals (Charleston, SC). Fresh diet was supplied to the rats daily, and all diets were stored at 4°C for no more than three weeks.

Experimental Animals

Sprague-Dawley rats were purchased from Sasco (Omaha, NE) and housed under standard conditions (room temperature 21 ± 3°C, 12:12-h light-dark cycle, 40 ± 5% relative humidity). Animals were caged in groups of four for the six-week study and in groups of five for the carcinogenesis study. They were checked daily and weighed weekly. For the six-week study and the scheduled sacrifice in the chronic study, rats were killed on proestrus, which has the characteristic peak estrogen secretion. Daily throughout the final two weeks of the experiment, rats were monitored for proestrus by cytology (25% nucleated, 75% cornified cells) (7). All procedures on live animals were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

Table 1. Experimental Diets[a]

	Control Diet	Lignin-Containing Diet			
		0.5%	1%	2.5%	5%
Corn oil	5.0	5.0	5.0	4.8	4.8
Casein	20.0	19.9	19.8	19.5	19.0
DL-Methionine	0.3	0.3	0.3	0.3	0.3
Glucose	15.0	14.9	14.8	14.6	14.2
Dextrin	50.0	49.7	49.5	48.8	47.6
Fiber (wood cellulose)	5.0	5.0	4.9	4.8	4.8
AIN mineral mix	3.5	3.5	3.5	3.5	3.3
AIN vitamin mix	1.0	1.0	1.0	1.0	1.0
Choline bitartrate	0.2	0.2	0.2	0.2	0.2
Lignin	0.00	0.5	1.0	2.4	4.8
Total	100.0	100.0	100.0	100.0	100.0

a: Control diet was formulated on the basis of recommendations of the American Institute of Nutrition (5,6). All diet ingredients except lignin were obtained from Teklad Test Diets (Madison, WI). Lignin AT was obtained from Westvaco Polychemicals (Charleston, SC).

Experimental Design

Two studies were conducted. The first was a short-term study to choose dietary lignin concentrations for the second carcinogenesis study. In the short-term study, graded levels of lignin (0%, 0.5%, 1%, 2.5%, and 5%) were fed beginning at six weeks of age to groups of eight female Sprague-Dawley rats. At the end of six weeks, rats were killed when they reached proestrus. The rats were anesthetized with phenobarbital, and the carotid artery was severed. Uteri were excised and weighed to assess estrogenic or antiestrogenic activity of the dietary lignin.

For the carcinogenesis experiment, rats were fed 2.5% lignin from six weeks of age until two days after MNU treatment, then 5% lignin until the termination of the experiment; a control group was fed a lignin-free diet. The lower dietary lignin concentration (2.5%) was fed initially because of a small but not statistically significant increase in body weight in rats consuming 5% lignin during the first two to three weeks in the short-term experiment. At 50 days of age, female Sprague-Dawley rats received a single tail vein injection of 5 mg MNU/100 g body wt iv in 0.03% acetic acid. Carcinogen- and vehicle-treated controls were included in the experimental design. All rats were randomly assigned; carcinogen-treated groups were assigned 49–50 rats; 5–11 were killed from each group at 4 and 12 weeks after MNU to evaluate uterine weights and circulating estradiol, the remaining 40–42 were evaluated for carcinoma development, and all survivors were killed 20 weeks after MNU. The non-carcinogen-treated groups were assigned 20 rats to be held in groups of 4–7 on each of the diets for 0, 4, 12, and 20 weeks after MNU injection for dietary controls. At 0, 4, and 12 weeks after MNU, randomly selected animals that had been monitored for proestrus as described above were killed for collection of samples. The selected animals were first anesthetized with phenobarbital, and blood was collected by cardiac puncture. Uteri were excised and weighed. Gross lesions were taken for histopathology.

The animals for the tumorigenesis study were monitored daily and weighed every other week. The mammary glands of the rats were palpated on a weekly basis for the presence of tumors. The location and size were measured in two perpendicular directions by a vernier caliper. Rats were killed when a lesion reached 2 cm, when the rats become moribund, or at 20 weeks after MNU, when the experiment was terminated. The rats were completely necropsied at death or sacrifice, and the mammary glands and any other gross lesions were excised and fixed in phosphate-buffered Formalin (10%, vol/vol). The uteri of rats surviving until 20 weeks after MNU were weighed, and samples were prepared for Northern blot analysis of creatine kinase B RNA. All tissues were processed

Table 2. Consumption of Control and 5% Lignin Diet in MNU-Treated Rats

Diet	Treatment[a]	Daily Consumption,[b,c] g
Control	Vehicle	14.1 ± 0.1†
5% Lignin	Vehicle	14.7 ± 0.1‡
Control	MNU	13.5 ± 0.1*
5% Lignin	MNU	14.3 ± 0.1†

a: Rats were treated by tail vein injection with 5 mg *N*-methyl-*N*-nitrosourea (MNU)/100 g body wt.

b: Values are means ± SE over 20 wk; $n = 21$ observations.

c: Diet and MNU effects were observed by ANOVA ($p < 0.05$). Individual differences were statistically significant by Student's *t*-test as follows: * < † < ‡ ($p < 0.001$).

by conventional histological techniques and stained with hematoxylin and eosin.

Serum estradiol (ICN Biomedical Diagnostics, Aurora, OH) was measured by radioimmunoassay. This assay measured total estradiol. RNA was prepared from frozen uteri by homogenization in TRIzol (GIBCO/BRL, Gaithersburg, MD). RNA (20 µg) was aliquoted for Northern blot analysis on 1.2% agarose and run at 5 V/cm. Samples were transferred to nitrocellulose (Schleicher and Schuell, Keen, NH), and a parallel gel was stained with ethidium bromide to verify the absence of degradation. The probe was prepared by labeling a creatine kinase B cDNA obtained from Brian Pentecost (Wadsworth Center for Laboratories and Research, Albany, NY) with ^{32}P by using a GIBCO/BRL random primers labeling kit (8). Hybridization was at 42°C overnight. Relative intensity on the blot was assessed by PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

Main effects of diet and MNU treatment on body and uterus weights, food consumption, circulating estradiol, and uterine creatine kinase B expression were evaluated statistically by analysis of variance (ANOVA). Comparisons of individual differences were made by Student's *t*-test, and $p < 0.05$ was used to assign significance. χ^2 tests were used to evaluate differences in tumor incidence.

Results

Food consumption (overall mean 16 ± 1 g), body weights (overall mean 220 ± 6 g), and uterine wet weights (overall mean 0.23 ± 0.02 g) of the rats fed graded levels of lignin (0%, 0.5%, 1%, 2.5%, and 5%) for six weeks did not differ between groups. Because there was a slight increase in body weight in the 5% lignin diet group during the first two to three weeks of the six-week trial, but weights of all the lignin-supplemented rats were comparable to the control rats at the end of the trial, we selected 2.5% dietary lignin for the initiation phase and 5% dietary lignin for the remainder of the tumorigenesis study.

Food consumption averaged over the carcinogenesis experiment is shown in Table 2. Consumption was reduced <5% in the MNU-treated groups ($p < 0.001$) and was elevated about 5% in the lignin-fed rats ($p < 0.001$). We expected the elevated intake in the lignin-fed animals because of the addition of 5% lignin, an undigested fiber, to the diet. Body weights for the four groups in the tumorigenesis study

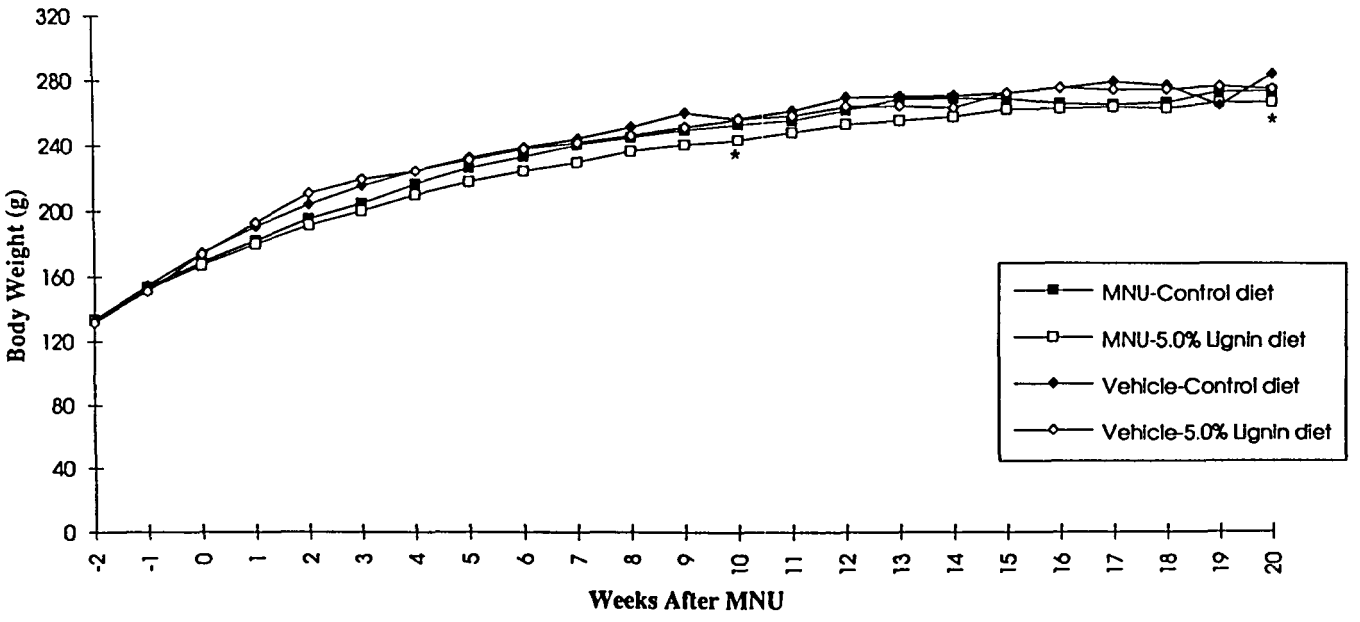

Figure 1. Body weights of rats in 5% lignin diet and *N*-methyl-*N*-nitrosourea (MNU) treatment groups. Values were statistically analyzed by ANOVA at 10 and 20 wk, and interaction of diet by MNU treatment was significant ($p < 0.05$). Individual values at these times were assessed by *t*-test (SE = 5–8 g). At 10 wk, body weight was significantly less in MNU-5% lignin diet group (*) ($n = 30$ rats) than in all other groups ($p < 0.05$) (MNU-control diet, $n = 30$ rats; vehicle-control diet, $n = 11$ rats). At 20 wk, MNU-5% lignin diet group weighed significantly less than vehicle-control diet group (*) ($n = 5$–7 rats/vehicle-treated group and $n = 10$–11 rats/MNU-treated group) ($p < 0.05$).

are shown in Figure 1. Statistical analysis at 10 and 20 weeks demonstrated reduced body weight in the MNU-lignin diet rats in comparison with all other groups at 10 weeks and in comparison with the vehicle-control diet group at 20 weeks ($p < 0.05$). Serum estradiol was measured at 0, 4, and 12 weeks after treatment with MNU. No differences were observed by carcinogen, time, or dietary treatment, and vehicle-control diet values averaged 97 ± 31 pg/ml at 0 week. Uterine weights were measured in rats killed at 0, 4, 12, and 20 weeks of the experiment, and MNU treatment of the control diet group increased tissue weight in comparison with the vehicle-control diet group at 4 weeks ($p < 0.03$) and in comparison with the vehicle-lignin diet group at 12 weeks ($p < 0.04$) (Table 3). Uterine weights did not differ between treatment groups at 20 weeks. Uterine creatine kinase B expression was determined at 20 weeks after MNU (Figure 2), and values were lowest in the MNU-lignin diet group [3.07 ± 0.09 (SE) PhosphoImager units] in comparison with the MNU-control diet (4.56 ± 0.31), the vehicle-control diet (4.99 ± 0.15), or the vehicle-lignin diet group (5.82 ± 1.09) ($p < 0.02$).

Mammary carcinoma development in the MNU-treated groups is shown in Figure 3. Dietary lignin did not alter the course of mammary cancer development in MNU-treated rats. Mammary lesions did not develop in the non-MNU-treated groups. Uterine endometrial adenocarcinoma and cervical squamous cell carcinoma (Figure 4) were observed only in the MNU-lignin diet group (4 of 40 effective rats, 10% incidence, killed at 11, 12, 13, and 20 weeks after MNU, average size = 0.7 × 0.5 × 0.5 cm) in comparison with the MNU-control diet group (0 of 42 effective rats) ($\chi^2 = 4.2$, $p < 0.05$). One MNU-control diet rat (killed at 15 weeks after MNU, 0.5 × 0.4 × 0.3 cm) had a uterine nodule identified as connective tissue and reactive epithelium indicating a repair/regeneration response. Uterine lesions did not develop in the non-MNU-treated groups.

Discussion

Prevention of breast carcinogenesis by components of cabbage was the focus of our previous research (1). Our initial studies determined that dried cabbage or dried collards incorporated at 5% or 10% of a control fat (5%) but not a high-fat (24.6%) diet inhibited mammary carcinogenesis by MNU (1). In the course of assessing the influence of the components from dried cabbage that were responsible for this effect, we incorporated the residue remaining after exhaustive extraction of dried cabbage at the level (3.2%) that would be expected to be present in the 5% cabbage diet. We compared these two diets with a control group, and the 5% cabbage and 3.2% residue diets were equally effective in the inhibition of MNU-induced mammary carcinogenesis. Thus the residue appeared to contain the effective chemopreventive agent. This residue was expected to consist of the dietary fiber, primarily lignin, cellulose, and other non-cellulose fibers.

Plant lignins are a potential precursor of the mammalian lignans previously shown to be produced by bacterial fermentation of a variety of plant foods (9). Cabbage was a poor substrate for lignan production compared with oil seeds but was comparable to other vegetables (9). Lignans are of particular interest in the present investigation because of the suggestion that they may be responsible, at least in part, for the inhibition of breast cancer by dietary fibers (10,11). It is possible that dietary lignins are converted by the intestinal bacteria to mammalian lignans, which are absorbed from the gut and excreted in the urine in relation to dietary intake of fiber (12,13).

The results of the present investigation demonstrate that lignin was not effective in the prevention of mammary carcinogenesis induced by MNU. Several reasons may account for the absence of cancer prevention of dietary lignin in the present study. First, we used wood lignin, and it is possible that this form of plant lignin is more insoluble and cannot serve as a precursor to the mammalian lignans. It is also possible that other lignans are not converted *in vivo* and that dietary cabbage lignin has preformed associated mammalian lignans. Furthermore, it is possible that cellulose, other non-cellulose fibers, or another trace material in our cabbage residue was responsible for the prevention of mammary carcinogenesis in our earlier investigation.

We used the MNU model for breast carcinogenesis. This model has relevance for our work because of the use of MNU in our previous studies (1). This model was particularly appropriate for our studies, because MNU does not

Table 3. Influence of 5% Lignin Diet on Uterine Net Weight in Rats[a,b]

		Weeks After MNU							
		0		4		12		20	
Diet	Treatment[c]	*n*	Uterine wt/100 g body wt	*n*	Uterine wt/100 g body wt	*n*	Uterine wt/100 g body wt	*n*	Uterine wt/100 g body wt
Control	Vehicle	5	0.28 ± 0.04	4	0.20 ± 0.01†	4	0.23 ± 0.03	7	0.24 ± 0.03
5% Lignin	Vehicle	5	0.23 ± 0.03	4	0.31 ± 0.07	5	0.22 ± 0.03†	6	0.22 ± 0.03
Control	MNU		NA	9	0.31 ± 0.04*	5	0.28 ± 0.01*	11	0.26 ± 0.03
5% Lignin	MNU		NA	9	0.26 ± 0.05	5	0.22 ± 0.04	10	0.24 ± 0.03

a: Values are means ± SE.

b: Interaction of MNU and diet was significant by ANOVA at 4 and 12 weeks ($p < 0.05$). Individual differences were statistically significant by Student's *t*-test as follows: at 4 wk (* > †; $p < 0.05$) and 12 wk (* > †; $p < 0.05$).

c: Rats were treated by tail vein injection with 5 mg MNU/100 g body wt.

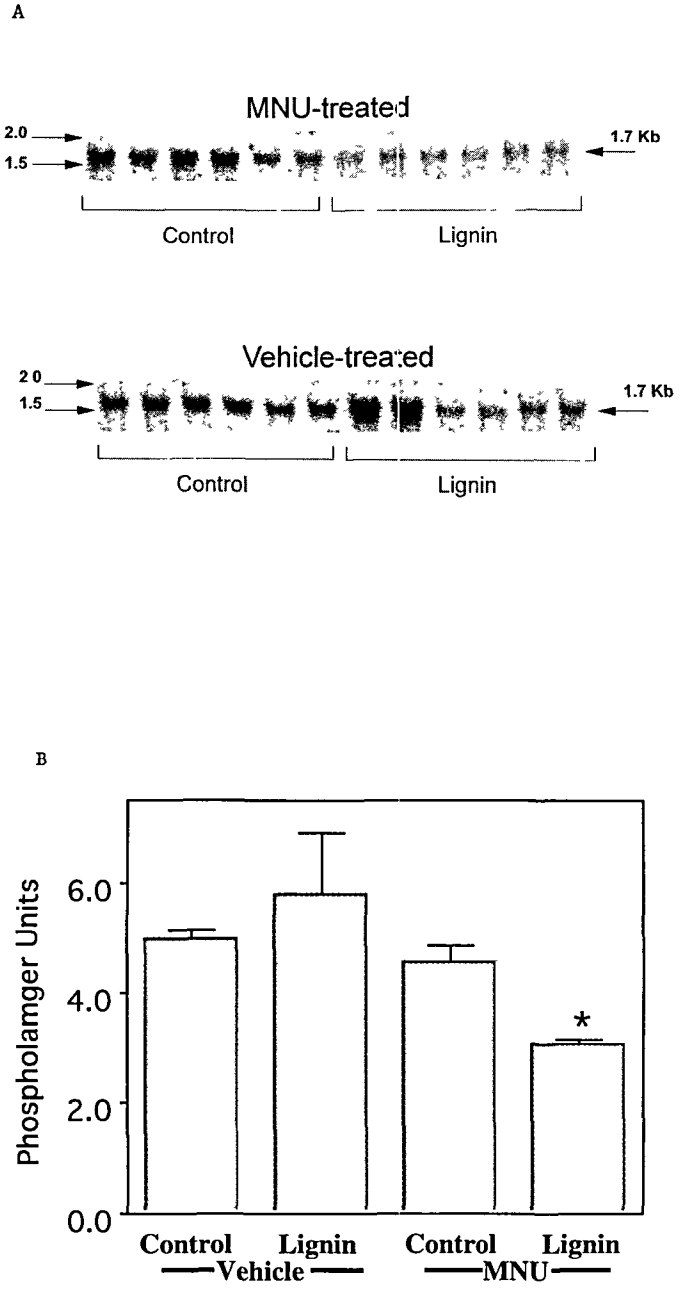

Figure 2. Creatine kinase B expression in uterus of rats at 20 wk after MNU or vehicle treatment in tumorigenesis study. In A, molecular weight markers are indicated by arrows at 2.0 and 1.5 Kb; 1.7-kDa arrow refers to approximate size of creatine kinase B mRNA. Each lane represents RNA from a single animal; $n = 6$ rats/treatment group. In B: histogram representing PhospholImager units (means ± SE). ANOVA demonstrated significant diet-by-MNU interaction ($p < 0.05$). Values were lower in MNU-lignin diet group (*) than in MNU-control diet, vehicle-control diet, or vehicle-lignin diet group ($p < 0.02$).

require metabolic activation. Thus lignin will presumably not act through altering the metabolism of MNU. This model had the added feature of inducing uterine carcinomas. For example, 22% of female Sprague-Dawley rats developed endometrial carcinoma at seven months after the first of three (4 mg/100 g body wt at 4 weekly intervals) injections of MNU (14). Interestingly, uterine endometrial adenocarcinomas were induced only in the rats fed dietary lignin in the present study, and carcinomas were observed in 10% of these rats at 20 weeks after a single injection of 5 mg MNU/100 g body wt. Thus dietary lignin increased susceptibility to the uterotrophic activity of MNU.

Previous studies involving the uterine endometrial carcinogenicity of MNU have generally used intravaginal administration of MNU to mice with or without administration of estrogens such as 17β-estradiol (15). However, increasing the survival of MNU-treated Sprague-Dawley rats by removal of developing mammary carcinomas allowed for the development of endometrial adenomatous hyperplasia and endometrial carcinomas in rats treated with multiple doses of MNU (3 weekly injections at 4 mg/100 g body wt iv) (14). However, these lesions were observed at 32 months after the first treatment with MNU rather than as early as 13 weeks after a single MNU application, as in the present report.

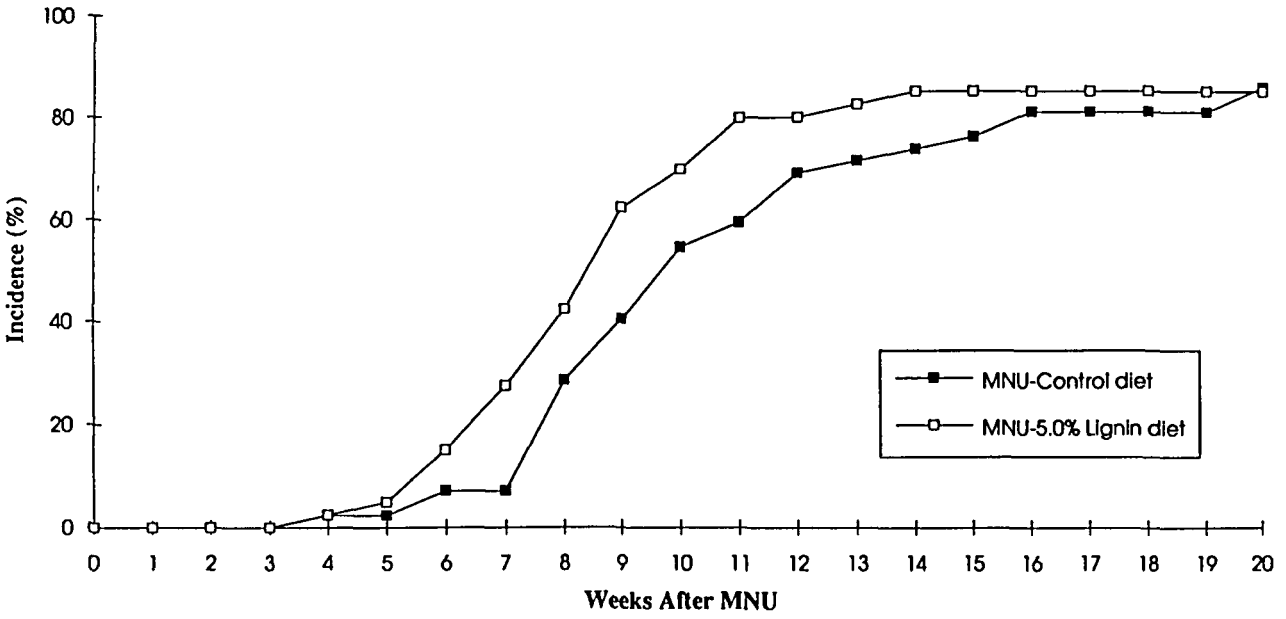

Figure 3. Incidence of mammary carcinoma in rats fed control or 5% lignin diet and treated with MNU. Statistical differences were not observed between groups ($p > 0.05$); $n = 42$ effective rats for control diet group and 40 rats for 5% lignin diet group.

Furthermore, multiple doses of MNU were associated with arrest of the estrous cycle and endometrial hyperplasia in another study with Sprague-Dawley rats (16). MNU treatment of female Sprague-Dawley rats also induced O^6-methylguanine and 7-methylguanine adducts in uterine DNA, and adduct levels were responsive to the stage of estrous cycle (17).

Natural estrogenic compounds such as coumestrol were demonstrated to have estrogenic activity and uterotrophic effects (18–20). It is possible that estrogenic activity of some absorbable component of lignin was responsible for the induction of uterine endometrial adenocarcinomas and cervical squamous cell carcinomas in the MNU-lignin diet group. However, the uterine weight data and the uterine creatine kinase B RNA expression did not provide evidence that the lignin fed in the present investigation had estrogenic activity. For example, expression of uterine creatine kinase B, an estrogen-responsive gene, was lowest in the group with the elevated uterine cancer, the MNU-lignin diet group.

These results demonstrated that 5% dietary wood lignin was not effective in the inhibition of MNU-induced mammary carcinogenesis in rats. However, incorporation of wood lignin into the diet resulted in the induction of endometrial adenocarcinomas. This research did not provide evidence that dietary wood lignin could be hydrolyzed to lignans.

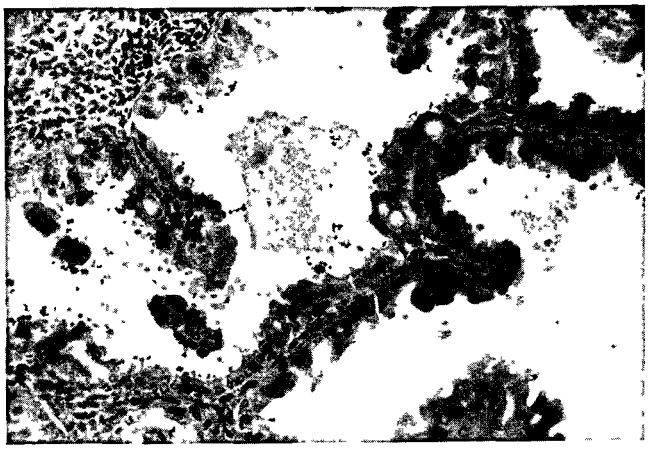

Figure 4. Photomicrograph of papillary adenocarcinoma component of a uterine endometrial carcinoma (hematoxylin and eosin-stained section, ×240).

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