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Inhibitory Effect of 13-*cis*-Retinoic Acid on Urinary Bladder Carcinogenesis Induced in C57BL/6 Mice by *N*-Butyl-*N*-(4-hydroxybutyl)-nitrosamine[1]

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ABSTRACT

The effect of 13-*cis*-retinoic acid on the induction of urinary bladder carcinoma by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (OH-BBN) was studied in male C57BL/6 mice. Animals received a total dose of either 90 or 140 mg of OH-BBN via gastric intubations of 7.5 or 10.0 mg of OH-BBN 2 times each week for 6 or 7 weeks, respectively. Seven days after the last OH-BBN intubation, animals were fed laboratory chow diet supplemented with either 200 mg of 13-*cis*-retinoic acid per kg or its placebo. Animals were killed at 6 months after the first carcinogen intubation. Highly invasive squamous and transitional cell carcinomas of the urothelium were found at autopsy. In the majority of these carcinomas, invasion of the bladder muscle wall by tumor cells had occurred. At the two dose levels of OH-BBN, feeding of 13-*cis*-retinoic acid reduced the incidence of both carcinomas and noninvasive papillomas, as well as the extent of neoplastic development in the urinary bladder. In mice receiving the lower dose of OH-BBN, the feeding of 13-*cis*-retinoic acid prevented the appearance of both squamous and transitional cell carcinomas with a reduction in incidence from 33 to 0% ($p < 0.01$). The results of this study indicate that 13-*cis*-retinoic acid reduced not only the severity of highly invasive urinary bladder carcinomas but also the incidence of such cancers.

INTRODUCTION

The synthetic retinoid, 13-*cis*-retinoic acid, has been shown to inhibit the development of urinary bladder cancer in female Wistar/Lewis rats that had been treated with the carcinogen 1-methyl-1-nitrosourea (6, 7) and in male Fischer 344 rats that had been treated with the carcinogen OH-BBN[3] (2). In these studies. 13-*cis*-retinoic acid inhibited the development of transitional and squamous cell carcinomas as well as the development of nonmalignant proliferative epithelial lesions of the rat urinary bladder. It was deemed appropriate to confirm the results of the rat studies by using another animal species, preferably in a model with tumors of even greater malignancy than those found in the rat. Since Bertram and Craig (1) have reported that OH-BBN induced highly invasive urinary bladder carcinomas in male C57BL/6 mice, a modification of their animal model was used to determine the effect of 13-*cis*-retinoic acid on the induction of highly invasive urinary bladder carcinoma.

MATERIALS AND METHODS

Male C57BL/6 mice (Simonsen Laboratories, Inc., Gilroy, Calif.), 6 to 7 weeks old and weighing 19 to 22 g at the time of the first carcinogen intubation, were housed in polycarbonate cages (5 mice/cage) in a room artificially illuminated for 12 hr each day and maintained at 22 ± 2°. All animals received Wayne Laboratory Chow 8604-00 (Allied Mills, Inc., Chicago, Ill.) and sterilized tap water *ad libitum*.

Animals were given either 7.5 or 10.0 mg OH-BBN [synthesized by the Chemistry Research Division, IIT Research Institute, Chicago, Ill., by a previously reported method (5)] via gastric intubation 2 times each week for 6 or 7 weeks, respectively, for a total dose of either 90 or 140 mg OH-BBN. OH-BBN was diluted with ethanol:water (20:80, v/v) so that each dose was contained in a volume of 0.2 ml. All control animals were intubated with 0.2 ml of the ethanol:water solution. Seven days after the last carcinogen intubation, mice were fed either a placebo diet or diet supplemented with 200 mg of 13-*cis*-retinoic acid per kg. The retinoid was added to the chow diet in the form of stable gelatinized beadlets, provided by Hoffmann-LaRoche Inc., Nutley, N. J. The placebo diet was formulated by blending gelatinized beadlets containing no retinoid into the chow diet. Animals were weighed weekly and observed twice daily for external signs of retinoid toxicity. All animals were killed at 6 months after the first carcinogen intubation.

At necropsy urinary bladders were distended with 10% buffered formalin, and a ligature was placed around the neck of the bladder to maintain proper distention. After fixation each urinary bladder was transected to yield an anterior and posterior cup-shaped area. Random serial transverse 5-μm paraffin sections from 2 different levels, as well as sections containing all grossly observed tumors, were cut from each of the 2 areas and stained with hematoxylin and eosin. Thus, each urinary bladder yielded 2 separate areas for histological evaluation from which the extent of neoplastic involvement of the bladder as a whole was evaluated. For diagnosis the slides from each individual animal were labeled as "anterior" and "posterior" and were

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[3] The abbreviation used is: OH-BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine.

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read as a set by 2 pathologists (Dr. P. J. Becci and Dr. R. A. Squire). The slides were randomized so that the nature of the treatment received by each individual animal was not known to the 2 pathologists evaluating the slides. The diagnosis of carcinoma was based on the presence of invasion of either underlying connective tissue or smooth muscle and of moderate to marked histological or cytological atypia.

The data were statistically analyzed for the one-sided hypothesis that a decrease in tumor incidence occurred in the retinoid-treated animals by the Fisher exact test (3) and by an extension of the Mantel-Haenszel procedure for χ^2 tests with 1 d.f. (4). Body weight data were evaluated by analysis of variance (8).

RESULTS AND DISCUSSION

The present study was conducted in order to determine the effect of 13-*cis*-retinoic acid against the induction of urinary bladder neoplasia in C57BL/6 mice. Bertram and Craig (1) have reported that male C57BL/6 mice were susceptible to OH-BBN-induced urinary bladder cancer. These investigators administered OH-BBN in the animal's drinking water; however, it is difficult to quantitate the amount of carcinogen ingested by each animal by this technique. We have overcome this disadvantage by administering a known amount of carcinogen via gastric intubation over a 6- to 7-week period. Furthermore, with this new dosing schedule, the stages of carcinogenesis (initiation and progression) may be separated. In our tumor model both the squamous and transitional cell carcinomas exhibited a short latency and were highly invasive (Figs. 1 and 3). The majority of the carcinomas exhibited invasion of the urinary bladder muscle wall by tumor cells (Figs. 2 and 4). The highly invasive nature of the carcinomas induced in mice by OH-BBN thus provided an animal model that is distinctly different from the previously used rat models (2, 6, 7) in which only subepithelial invasion was noted.

Table 1 shows the distribution of urinary bladder carcinomas and noninvasive papillomas among the various groups. Since there was excellent agreement in the diagnoses between the 2 pathologists, the results were averaged. The data indicate that feeding diet supplemented with 13-*cis*-retinoic acid inhibited the development of neoplasia of the urinary bladder epithelium.

The retinoid had a marked effect on cancer incidence in the groups that were given the lower dose of OH-BBN and then fed either the placebo diet (Group A) or diet supplemented with 13-*cis*-retinoic acid (Group B). The incidence of bladder neoplasms was reduced from 38% (Group A) to 5% (Group B) ($p < 0.05$), while 13-*cis*-retinoic acid prevented the appearance of carcinomas. The combined incidence of both squamous and transitional cell carcinoma was reduced from 33% (Group A) to 0% (Group B) ($p < 0.01$), whereas the incidence of squamous cell carcinoma alone was reduced from 25% (Group A) to 0% (Group B) ($p < 0.05$). Thus, under the conditions of the present study, the feeding of 13-*cis*-retinoic acid prevented the appearance of urinary bladder cancer induced in mice given the lower dose of OH-BBN. Furthermore, the retinoid reduced the extent of neoplastic development in the bladders of the mice given the lower dose of OH-BBN. The percentage of areas with bladder neoplasms, squamous or transitional cell carcinoma, or squamous cell carcinoma alone was reduced from 23% to 3% ($p < 0.01$), 21% to 0% ($p < 0.01$), and 17% to 0% ($p < 0.05$), respectively.

In the groups that were given the higher dose of OH-BBN (Groups C and D), the feeding of the retinoid reduced the incidence of carcinoma and noninvasive papillomas as well as the extent of neoplastic development in the urinary bladder epithelium. However, only the reduction in areas with bladder neoplasms from 36% (Group C) to 18% (Group D) attained statistical significance ($p < 0.05$).

No lesions were observed in the animals treated with the ethanol:water solvent and fed either a placebo diet (Group E) or diet supplemented with 13-*cis*-retinoic acid (Group F). No calculi, mineralization, or parasites were noted in the urinary bladders.

In previous studies (2, 6, 7) 13-*cis*-retinoic acid was shown to inhibit the development of chemically induced urinary bladder cancer in rats. Although in those studies a statistically significant reduction in the severity of the lesions was evident, a significant decrease in cancer incidence was not found. However, in the present study in which C57BL/6 mice were used, 13-*cis*-retinoic acid not only reduced the severity of these highly aggressive and

Table 1

Effect of 13-cis-retinoic acid: incidence of bladder neoplasms in each treatment group

Group	No. of mice	Total dose of OH-BBN (mg)	Dose of 13-*cis*-retinoic acid (mg/kg diet)	Total bladder neoplasms[a]		Transitional and squamous cell carcinoma		Squamous cell carcinoma	
				No. of mice	No. of bladder areas	No. of mice	No. of bladder areas	No. of mice	No. of bladder areas
A	24	90	None	9 (38)[b]	11 (23)	8 (33)	10 (21)	6 (25)	8 (17)
B	19	90	200	1 (5)[c]	1 (3)[d]	0 (0)[d]	0 (0)[d]	0 (0)[c]	0 (0)[c]
C	22	140	None	12 (55)	16 (36)	10 (45)	14 (32)	6 (27)	9 (20)
D	25	140	200	8 (32)	9 (18)[c]	7 (28)	8 (16)	5 (20)	6 (12)
E	10	None	None	0	0	0	0	0	0
F	10	None	200	0	0	0	0	0	0

[a] Bladder neoplasms included carcinomas and noninvasive transitional cell papillomas.
[b] Numbers in parentheses, percentage.
[c] Significantly different from respective control; $p < 0.05$.
[d] Significantly different from respective control; $p < 0.01$.

invasive chemically induced urinary bladder carcinomas but also prevented them from occurring. These findings assume added importance since the prevention of urinary bladder carcinoma by 13-*cis*-retinoic acid was not accompanied by any signs of toxicity or a reduction in body weight gain caused by the retinoid.

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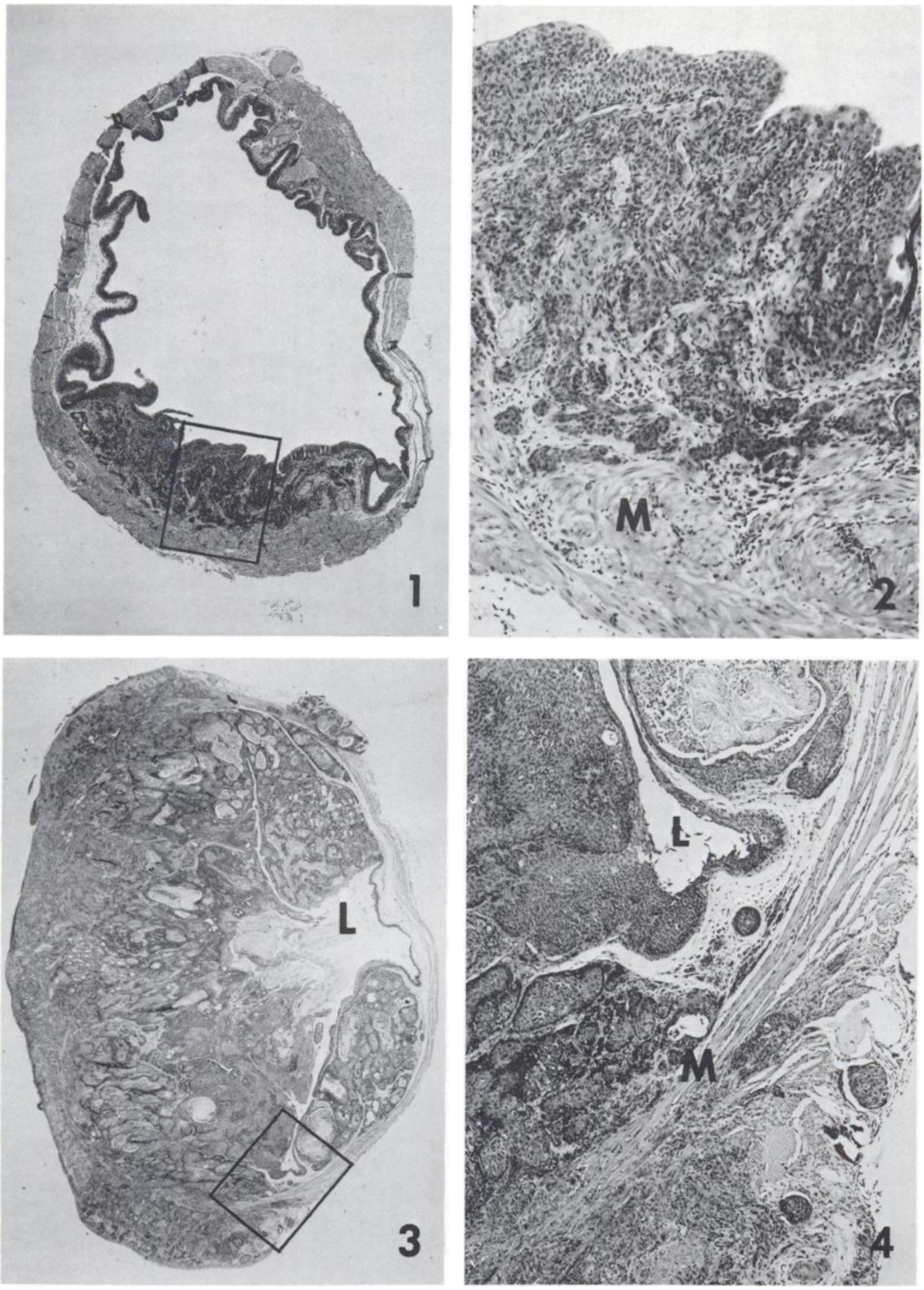

Fig. 1. Transitional cell carcinoma of the urinary bladder. H & E, × 20.

Fig. 2. A higher magnification of a portion of the tumor seen in Fig. 1 (*area inside square*). Nests of tumor cells extend to the muscle layer (*M*). H & E, × 100.

Fig. 3. Squamous cell carcinoma of the urinary bladder. *L*, bladder lumen. H & E, × 10.

Fig. 4. Higher magnification of a portion of the tumor seen in Fig. 3 (*area inside square*). *L*, bladder lumen. Tumor cells have penetrated through the muscle layer (*M*). H & E, × 50.