HISTOGENESIS OF URINARY BLADDER TUMORS INDUCED BY N-BUTYL-N-(4-HYDROXYBUTYL)NITROSAMINE IN RATS

(Plates LXIV~LXVIII)

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Synopsis

Histogenesis of urinary bladder tumors in Wistar strain male rats after administration of N-butyl-N-(4-hydroxybutyl)nitrosamine was studied by light microscope, electron microscope, and autoradiography. N-Butyl-N-(4-hydroxybutyl)nitrosamine administration resulted in a selectively high incidence of urinary bladder tumors. Hyperplasia of the urinary bladder epithelium was always observed within 8 weeks and papilloma within 16 weeks, and cancer developed within 20 weeks in 84.7% of the animals. Histologically, the carcinoma, a transitional cell carcinoma, closely resembled those produced in the urinary bladder on administration of other chemical carcinogens. Electron microscopically, tonofibriles were observed in the tumor tissue. Autoradiographically, a good correlation was found between the distribution of cells labeled with tritiated thymidine and the histologic grade of malignancy of the urinary bladder tumors. These results showed that histological changes in the urinary bladder epithelium increase in frequency and extent with the period of N-butyl-N-(4-hydroxybutyl)nitrosamine administration. These results also suggest that papillomas are precursors of bladder cancer in rats administered N-butyl-N-(4-hydroxybutyl)nitrosamine.

There have been many reports1,2,4~13,17) on the carcinogenic effect of aromatic amines on the urinary system and particularly on the urinary bladder of experimental animals.

After the first report of the carcinogenic activity of N-nitrosodimethylamine, many nitrosamines have been prepared and assessed as carcinogens.15,21,22) A number of nitrosamines induced tumors with a high degree of organ specificity, but changes in the chemical structure of nitrosamine compounds, their total dose, and route of administration frequently alter the organs affected.15,22) Druckrey et al.14) reported that when N-butyl-N-(4-hydroxybutyl)nitrosamine was given continuously in the drinking water for about 40 weeks, urinary bladder tumors, a squamous epithelium carcinoma, were induced in all the experimental animals.

This paper reports studies on the histogenesis of urinary bladder tumors in rats induced by N-butyl-N-(4-hydroxybutyl)nitrosamine. The correlation between the macroscopic appearance of the lesions and observations by light microscopy, electron microscopy, and autoradiography was also examined.

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MATERIALS AND METHODS

Wistar strain male rats (Fuji Animal Farm, Tokyo), average body weight 150~160 g, were used. One hundred and twenty-seven rats were divided into 6 groups. The carcinogen, N-butyl-N-(4-hydroxybutyl) nitrosamine (Chugai Pharmaceutical Co., Tokyo) was given as 0.05% solution in the drinking water. All the rats received commercial stock diet from CLEA Japan Inc., Tokyo.

**Group 1** Thirteen rats were given water supplemented with N-butyl-N-(4-hydroxybutyl) nitrosamine for 20 weeks. They were killed and examined by light microscopy, electron microscopy, and autoradiography after 20 weeks.

**Group 2** Eighteen rats were given drinking water supplemented with N-butyl-N-(4-hydroxybutyl) nitrosamine for 16 weeks and then water without N-butyl-N-(4-hydroxybutyl) nitrosamine for 4 weeks. Five animals were killed for light microscopic and autoradiographic studies after 16 weeks. Eight animals were killed after 20 weeks for light microscopic observations.

**Group 3** Twenty-four rats were given drinking water supplemented with N-butyl-N-(4-hydroxybutyl) nitrosamine for 12 weeks and then water without N-butyl-N-(4-hydroxybutyl) nitrosamine for 8 weeks. Five animals were killed for light microscopic and autoradiographic studies after 12 weeks. Six animals were killed after 16 weeks and 11 after 20 weeks for light microscopic observations.

**Group 4** Thirty rats were given drinking water supplemented with N-butyl-N-(4-hydroxybutyl) nitrosamine for 8 weeks and then water without N-butyl-N-(4-hydroxybutyl) nitrosamine for 12 weeks. Six rats were killed for light microscopic and autoradiographic studies after 8 weeks. Six animals were killed after 12 weeks, 7 after 16 weeks, and 10 after 20 weeks for light microscopic observations.

**Group 5** Thirty-six rats were given drinking water supplemented with N-butyl-N-(4-hydroxybutyl) nitrosamine for 4 weeks and then water without N-butyl-N-(4-hydroxybutyl) nitrosamine for 16 weeks. Six rats were killed for light microscopic and autoradiographic studies after 4 weeks. Six rats were killed after 8 and 12 weeks, 5 after 16 weeks, and 11 after 20 weeks for light microscopic observations.

**Group 6** Six rats were given drinking water without N-butyl-N-(4-hydroxybutyl) nitrosamine for 20 weeks. All the rats were killed for light microscopic and autoradiographic studies after 20 weeks.

Before the experiment, 3 non-treated rats were sacrificed for light microscopic and autoradiographic studies on the urinary bladder.

Rats were housed three to a wire cage in an air-conditioned room kept at 24° and weighed weekly. All the rats were killed with ether. None of the rats dying before sacrifice was used. The liver and both kidneys were weighed and samples were taken for histological studies. Urinary bladders were punctured at the ureter, and 0.5 ml of 10% buffered formaldehyde solution or Bouin’s solution was then injected into the bladder.

For light microscopic studies, tissues were fixed and stained with Hematoxylin and Eosin, van Gieson, Mallory stain, and periodic acid-Schiff stain. For electron microscopic studies, tissues were fixed in buffered glutaraldehyde and OsO₄, and embedded in Epon resin. Sections were cut on a Potter-Blum microtome or an LKB ultratome with a glass knife. They were mounted on Formvar-coated copper specimen grids and
doubly stained with aqueous uranyl acetate followed by lead citrate. Sections were examined with Hitachi Model HU-11DS electron microscope. For autoradiographic studies, rats were given ³H-thymidine (specific activity, 5.0 Ci/mM; Radiochemical Centre, Amersham, England), 1 µCi/g body weight, intraperitoneally, exactly 1 hr before sacrifice. Sections of the urinary bladder were fixed in Bouin’s solution and embedded in paraffin. Autoradiographs were prepared as described previously by coating the sections with Kodak NTB-2 nuclear track emulsion, and exposing them for 3 weeks at 4°C. The preparations were treated with Kodak D-19B developing solution and then stained with Ehrlich’s Hematoxylin and Eosin. Fig. 1 summarizes the scheme of the experiments.

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Sacrificed for: light microscopic observation (L), autoradiograph observation (A), electron microscopic observation (E),

- BBN in water
- No BBN in water

Fig. 1. Scheme of experiments on urinary bladder carcinogenesis in rats treated with N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN)

**RESULTS**

The mean duration of the experiment in each group and the average changes in body, liver, and kidney weights of the rats in each group are summarized in Table I.

All the rats gained weight. In general, the changes were confined to the urinary bladder and no remarkable changes were seen in the liver, kidney, lung, or gastrointestinal tract.
Table I. Changes in Body, Liver, and Kidney Weight in Rats treated with N-Butyl-N-(4-hydroxybutyl)nitrosamine (BBN)

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<th>Group No.</th>
<th>Period for drinking water (weeks) with BBN</th>
<th>Effective No. of rats</th>
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Findings of the Urinary Bladder

Three kinds of changes were distinguished in the bladder.

Hyperplasia: Thickening of the bladder epithelium (Photo 1). The changes in most cases were strictly localized. Oval cell infiltration was occasionally seen in the sub-epithelial layer of the bladder (Photo 2).

Papilloma: Extensive epithelial proliferation, with a tendency to formation of papilloma (Photo 3). Cellular irregularity was slight and a few mitotic figures were noticed in the proliferative areas of the bladder epithelium (Photo 4).

Cancer: The tumor was usually of the transitional cell type (Photo 5). Squamous metaplasia and mitotic figures were frequently seen (Photo 6). Cystic degeneration, hemorrhage, and inflammatory changes in the stromal tissues were also noticed in this category (Photo 7).

Light Microscopic Findings

Group 1: Papillomatous changes in the urinary bladder epithelium were seen in all the animals. Microscopically, all the specimens showed hyperplasia and papilloma of the urinary bladder epithelium. Eleven of the 13 rats (84.6%) in this group developed cancer. Two of these 13 showed muscular invasion of the bladder wall (Photo 8). No metastases were observed.

Group 2: After 16 and 20 weeks, all the rats in this group had developed hyperplasia and papilloma of the urinary bladder epithelium. After 16 weeks, 3 of the 5 rats (60.0%) had developed cancer of the bladder. After 20 weeks, 5 of the 8 rats (62.5%) had developed cancer of urinary bladder. No invasion of the bladder wall was seen.

Group 3: After 12, 16, and 20 weeks, all the rats in this group had developed hyperplasia of the bladder epithelium. The incidences of papilloma were as follows: 4/5 (80.0%) after 12 weeks, 5/6 (83.3%) after 16 weeks, 11/11 (100.0%) after 20 weeks. The incidences of bladder cancer were as follows: 1/5 (20.0%) after 12 weeks, 2/6 (33.3%) after 16 weeks, 8/11 (72.7%) after 20 weeks. No metastatic changes were seen in rats with bladder cancer.

Group 4: After 8 and 12 weeks, all the rats developed hyperplasia of the bladder epithelium. After 16 weeks, 6 of 7 rats (85.7%) and after 20 weeks, 9 of the 10 rats (90.0%) developed hyperplasia of the bladder epithelium. The incidences of papilloma in the bladder were as follows: 2/6 (33.3%) after 8 weeks, 3/6 (50.0%) after 12 weeks, 4/6 (57.1%) after 16 weeks, 10/10 (100.0%) after 20 weeks. After 20 weeks, 2 of the 10 rats (20.0%) developed cancer of the bladder. No metastatic changes were seen in this group.

Group 5: The incidences of hyperplasia of the mucosa of the bladder were as follows: 1/6 (16.7%) after 4 weeks, 2/6 (33.3%) after 8 weeks, 2/6 (33.3%) after 12 weeks, 2/5 (40.0%) after 16 weeks, 6/11 (54.6%) after 20 weeks. Papilloma was seen only in one of the 11 rats in this group after 20 weeks. No other rats developed papilloma or cancer of the bladder.

Group 6: No rats developed papilloma or cancer of the bladder. No histological abnormalities were observed.

Other Organs

No marked macroscopic or microscopic changes were seen in the liver, kidney, or lungs.
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<th>Effective No. of rats without BBN</th>
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The histological findings in the bladder of all the experimental animals are summarized in Table II and are shown graphically in Fig. 2.

**Electron Microscopic Findings**

In cancer cells in the bladder, the nuclei were oval or round with few indentations like normal bladder cells. Nuclear hypertrophy and nucleolar hypertrophy and complexity were marked and nucleoli were frequently multiple (Photo 9). In cancer cells, the number of ribosomes was usually increased. The number of mitochondria was normal (Photo 10). Frequently, the cytoplasm in tumors showed degenerative changes. Some tumor cells contained tonofibriles (Photo 11). These tonofibriles were abundant but were also found in isolated cells although the tumor appeared to be typical papillary carcinoma by light microscopy.

**Autoradiographic Findings**

Autoradiographs were examined for changes in the distribution of proliferating cells in response to different kinds of changes in the bladder. A few labeled cells were seen in areas of hyperplasia of the bladder epithelium, but most of the labeled cells were in the basal cell layer (Photo 12). The oval cells in the subepithelial layer of the bladder were heavily labeled (Photo 13). After 12 to 20 weeks, many labeled cells were observed in papillomatous areas, but most of them were still in the basal cell layer (Photo 14). After 16 to 20 weeks, many labeled cells were seen in the area of cancer. All the cell layers such as the superficial, middle, and basal layers were densely labeled (Photo 15).

**DISCUSSION**

The present results essentially confirm those of Druckrey *et al.* on the carcinogenic effect of N-butyl-N-(4-hydroxybutyl)nitrosamine on rat urinary bladder. There has been much work on urinary bladder cancer, but cancer has seldom been produced in the bladder of small experimental animals.
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Histologically and histochemically, N-butyl-N-(4-hydroxybutyl)nitrosamine-induced tumors, papillomas, and carcinomas of the bladder were very similar to those produced on administration of other chemical carcinogens. The appearance of tumor cells under the electron microscope also resembled that of cells in other chemically induced tumors and also human bladder tumors. The tonofilaments seen in tumor cells induced by N-butyl-N-(4-hydroxybutyl)nitrosamine were quite similar to those in rat bladder tumors induced by N-2-fluorenylacetamide. Previously, Veenema et al. and Battifora et al. found a good correlation between the percentage of cell nuclei labeled with tritiated thymidine and histological grade of malignancy in human urinary bladder cancer. Our present autoradiographic data on the rat agree with theirs. Cancer induced by N-butyl-N-(4-hydroxybutyl)nitrosamine first appeared in the bladder epithelium 12 weeks after treatment in our experiments. However, Druckrey et al. found a high incidence of cancer in rat bladder after treatment with N-butyl-N-(4-hydroxybutyl)nitrosamine for about 1 year. In our work, the first characteristic changes in the bladder epithelium on treatment with N-butyl-N-(4-hydroxybutyl)nitrosamine were hyperplasia and formation of papilloma. Hyperplasia was always observed after 8 weeks and papilloma after 16 weeks, while cancer was seen in 84.7% of the animals after 20 weeks. These results showed that the histological changes of urinary bladder epithelium increase in number and extent during treatment with N-butyl-N-(4-hydroxybutyl)nitrosamine.

Our results show a large difference in the incidence of bladder cancer in the groups treated with N-butyl-N-(4-hydroxybutyl)nitrosamine for 8 and 12 weeks. It would be interesting to analyze the alterations of the bladder epithelium from the time when N-butyl-N-(4-hydroxybutyl)nitrosamine-containing water was discontinued until the appearance of cancer. When rats were given N-butyl-N-(4-hydroxybutyl)nitrosamine-containing water for 12 weeks, the incidence of cancer was 72.7% while when given N-butyl-N-(4-hydroxybutyl)nitrosamine-containing water for only 8 weeks, the incidence of cancer was only 20.0%. These results suggest that two types of precursor lesion or hyperplasia may be present, reversible and irreversible, but two types could not be distinguished by light or electron microscopy or by autoradiography.

The effects of many other chemical carcinogens on the urinary bladder have been studied in vivo and in vitro. Recently, Okajima et al. observed the synergistic effect of L-tryptophan and N-butyl-N-(4-hydroxybutyl)nitrosamine on bladder carcinogenesis in rats, but the fate of N-butyl-N-(4-hydroxybutyl)nitrosamine is still not clear.

The relationship between histogenesis of urinary bladder cancer and the metabolism of N-butyl-N-(4-hydroxybutyl)nitrosamine in vivo requires further study.

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education (No. 9313, 1968) and by a grant from the Tokyo Biochemical Research Foundation which are gratefully acknowledged.

(Received January 13, 1969)
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EXPLANATION OF PLATES LXIV–LXVIII


Photo 4. Higher magnification of Photo 3, showing a few nuclear irregularities. H-E. ×160.


Photo 6. Higher magnification of Photo 5, showing mitotic figure and nuclear irregularity. H-E. ×160.


Photo 9. Low-power electron micrograph of area of cancer in a rat treated with N-butyl-N-(4-hydroxybutyl)nitrosamine for 20 weeks. Tumor cells with oval or round nuclei and irregular large nucleoli are seen. ×2,100.

Photo 10. Electron micrograph of area of cancer. Cytoplasm contains lysosomes and increased number of ribosomes. ×7,500.


Photo 12. Autoradiograph of urinary bladder epithelium in a rat treated with N-butyl-N-(4-hydroxybutyl)nitrosamine for 4 weeks, showing a few labeled cells in a basal cell layer. H-E. ×160.


H-E= Hematoxylin and Eosin stain