Distinction by Concanavalin A Agglutination between Ulceration and Repair of Rat Bladder Epithelium Induced by Freezing or Cyclophosphamide and the Effect of Sodium Saccharin

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ABSTRACT

Agglutination of rat urinary bladder epithelial cells by concanavalin A (Con A) has been reported to be an early marker of bladder carcinogenesis. Ulceration of the bladder, induced by cyclophosphamide (CP) or freezing, followed by sodium saccharin in the diet results in the induction of bladder cancer. In the present studies, the agglutination of rat urinary bladder epithelial cells by Con A was shown to be increased during the regenerative hyperplasia following ulceration induced by i.p. CP injection, but it returned to normal levels by Day 21 when the preparative process was nearly complete. This effect correlated quantitatively with the dose of CP. However, if CP administration was followed by sodium saccharin in the diet beginning 14 days after the injection, the agglutination of bladder cells by Con A persisted. In contrast, agglutination of bladder cells by Con A during regenerative hyperplasia following ulceration induced by freezing was not increased whether sodium saccharin was fed or not. These results indicate that Con A agglutination distinguishes between the regenerative hyperplasia induced by CP or freezing, even though either method followed by sodium saccharin in the diet results in bladder cancer in the rat.

INTRODUCTION

Several potent urinary bladder-specific carcinogens have been identified in experimental animals (15), most notably N-butyl-N-(4-hydroxybutyl)nitrosamine administered in the drinking water, N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide fed in the diet, and N-methyl-1-nitrosourea instilled intravesically (4). In contrast, sodium saccharin is a weak bladder carcinogen but has considerable promoting activity when administered after a low dose of one of the potent bladder carcinogens (4, 8). However, sodium saccharin, fed immediately after ulceration induced by freezing or by an i.p. injection of CP, or beginning 2 weeks later, resulted in persistence of the resulting regenerative nodular and papillary hyperplasia, persistence of the increased proliferative rate, and eventually the appearance of bladder tumors, even without prior N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide initiation (5, 13).

Kakizoe et al. (9, 10) demonstrated that the agglutination of rat urinary bladder epithelial cells by Con A increased after the administration of carcinogens. This increase occurred early, even before morphological changes, light microscopic or ultrastructural, became evident. In contrast, Con A agglutination was not increased by sodium saccharin feeding unless it was fed after exposure to a carcinogen (11). To further evaluate the specificity of this process for carcinogenesis, we examined the agglutinability of rat bladder epithelial cells during regenerative hyperplasia following ulceration and the effect of sodium saccharin on this response.

MATERIALS AND METHODS

Five-week-old inbred male Fischer 344 rats (157 ± 9 g; Charles River Breeding Laboratories, Wilmington, Mass.) were used and maintained 5/cage in a room at 24° and 50% humidity on a 12-hr light-dark cycle. Food and water were available ad libitum. Sodium saccharin (synthesized by the Maumee procedure; Sigma Chemical Co., St. Louis, Mo.) was mixed in a diet (Charles River rat Chow) at a level of 5.0% by weight and pelleted. The pelleted form of the Chow without sodium saccharin was fed as control diet. CP (Mead Johnson & Co., Evansville, Ind.) dissolved in sterile water (The Dexter Co., Chagrin Falls, Ohio) and injected into the peritoneal cavity at a dose of 100 mg/kg body weight. The concentration of the CP solution was adjusted so that 0.5 to 0.7 ml was injected, and each rat received only a single injection.

Freeze ulceration of the urinary bladder was performed by the method of Shirai et al. (17), with the rats under light Nembutal (Abbott Laboratories, North Chicago, Ill.) anesthesia (50 mg/ml, 5 mg/kg body weight). A steel rod, 5 mm in diameter, frozen in dry ice-acetone, was applied twice in the same place to the serosal surface of the ventral portion of the urinary bladder for 2 sec each time, with 5 sec between each application. Sham operation of the urinary bladder was done with the rats under Nembutal anesthesia; the ventral portion was manipulated twice with a forceps for 2 sec each time, with 5 sec between each manipulation.

In the first experiment, the rats were divided randomly into 4 groups treated as illustrated in Chart 1. The rats were numbered sequentially within each group. In Groups 1 to 3, CP injection, freeze ulceration, and sham operation, respectively, were followed for 2 weeks with rats being fed control diet, and then sodium saccharin was administered as 5% of the diet until the end of the experiment. In contrast, in Group 4, CP injection was followed by control diet alone for the entire experiment. More than the projected number needed were begun in each group in case of mortality during the experiment. In Group 1, 3 rats died due to toxicity, but none died in the other groups. No significant difference in growth or diet consumption was noted between groups of rats. In each group, 5 rats (the lowest numbered rats remaining) were sacrificed at each of various times up to Day 70 after the beginning of the experiment, as shown in Chart 1.

In the second experiment, 25 rats were divided randomly into 5 groups. The groups were given injections i.p. once with 0, 10, 25, 50, or 100 mg of CP per kg of body weight, respectively. All rats in the second experiment were fed control diet and sacrificed 6 days after CP injection.

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5 The abbreviations used are: CP, cyclophosphamide; Con A, concanavalin A; α-MM, α-methyl mannoside.

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Con A Agglutination of Urothelial Cells

Chart 1. Experimental design for Experiment 1. [Control diet; freeze ulceration; CP (100 mg/kg body weight, i.p.) injection; sham operation.]

For the agglutination test of rat bladder epithelial cells by Con A, we modified the method of Kakizoe et al. (9). Briefly, the urinary bladder was removed with the rat under Nembutal anesthesia, then immediately everted, washed, and incubated in 0.1 M phosphate-buffered saline, pH 7.4, for 15 min at room temperature. It was sonicated in an ultrasonic cuvet washer (Millipore, Bedford, Mass.) at room temperature for 20 sec, and the mucosal surface was then squashed by agitating with a loose-fitting Potter-Elvehjem homogenizer in a plastic test tube. The epithelial cells isolated from the 5 rats in each group were combined and collected by centrifugation (IEC HN-SII centrifuge; Damon/IEC Division), first at 600 rpm for 3 min to remove tissue fragments and then at 2000 rpm for 10 min. Agglutination was assayed in a final volume of 80 μl of phosphate-buffered saline containing 2 to 5 × 10⁶ cell/ml and 0, 200, or 400 μg of Con A (Sigma Chemical Co.) per ml with or without 100 μg of α-MM (Sigma Chemical Co.) per ml in a round-bottomed (96 wells) microtitration multiwell plate (Flow Laboratories, McLean, Va.) During incubation, it was agitated once by a mixer (Vortex-Genii; Fisher Scientific Co., Bohemia, N.Y.). After incubating in an automatic CO₂-water-jacketed incubator (Napco 6300; National Appliance Co., Portland, Oreg.) for 30 min at 37°, the number of aggregates of 3 or more cells per 200 single cells or aggregates were counted in a hemocytometer. Numbers of aggregates were determined by the subtraction of the scores of the reactions with neither Con A nor α-MM present from the scores of the reactions with Con A present, with or without α-MM.

The references (in "Results" and "Discussion") to the histological sequence of events following freeze ulceration or CP are based on previous studies in our laboratory (5-7, 13) which have been consistent between repeated experiments.

RESULTS

Con A agglutination increased during the regenerative hyperplasia following a single injection of CP (100 mg/kg), reaching peak levels by Day 6 after injection (Chart 2). The numbers of aggregates on Days 1 and 3 were 16.3 ± 4.7 and 19.0 ± 2.6, respectively. If continued on control diet (Group 4), the level of agglutination returned to control levels within 21 days after injection. However, if sodium saccharin was fed beginning 14 days after injection, the extent of Con A agglutination increased, remained elevated through 42 days after injection, but returned to control levels by Day 70.

In contrast, Charts 3 and 4 (Groups 2 and 3, respectively) show that Con A agglutination did not increase during the regenerative hyperplasia following freeze ulceration or after sham operation. Also, there was no increase when sodium saccharin was added to the diet 14 days after ulceration except for a small increase at Day 42 of the experiment.
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The relation between the dose of CP and the agglutination of bladder epithelial cells by Con A is illustrated in Chart 5. There was essentially no difference in the number of cellular aggregates between the 2 Con A concentrations evaluated. Agglutination was increased significantly at doses of CP at 25 mg/kg and higher. However, the increasing rate of aggregation appeared to plateau between 50- and 100-mg/kg doses.

DISCUSSION

Ulceration of the urinary bladder, whether induced by freezing or CP, results in a marked regenerative hyperplasia with subsequent repair (6, 7). The kinetics of this process is related to the extent of the ulceration (i.e., time of freezing or dose of CP), but generally, the bladder epithelium returns to normal microscopically, ultrastructurally, and with respect to cell turnover rates within 3 to 4 weeks after the inciting event (6, 7). These regenerative phenomena in rats are reversible, and no tumors result even if the rats survive up to 2 years (5). However, if sodium saccharin is administered during the regenerative process and continued for the remainder of the rat’s life, a significant incidence of bladder tumors results (5).

CP induces multifocal, small ulcerations of the bladder, and the freezing technique induces only a single, large ulcer. The consequent proliferative response in the epithelium, however, is diffuse in both situations, although most intense around the ulcer. The effect of sodium saccharin administration (5), the transmission and scanning electron microscopic appearance (6, 7), the histochemical (3) and biochemical (14) determinations so far measured, and the ability to stimulate angiogenesis (20) have been similar for the regenerative hyperplasia in response to either of the inciting stimuli. Con A agglutination, however, appears to readily distinguish between them. It is unlikely that this difference is due to a greater proliferation following CP than after freezing, since a positive response was observed even after lower doses of CP, doses at which ulceration does not occur (6). Similar to the results with bladder carcinogens reported by Kakizoe et al. (9, 10), Con A agglutination increased markedly following CP injection i.p. Similar to these bladder carcinogens, CP is mutagenic after metabolic activation (12). Although CP is not carcinogenic to the bladder at the doses examined in our experiments (2, 5, 8), at considerably lower doses, it has been reported to have weak carcinogenic activity for the rat bladder (16). Also, it has been shown to be a bladder carcinogen in humans (21). Its lack of carcinogenicity at the doses evaluated in the present experiments might be related to the marked cytotoxic effect it has on urothelial cells (19), destroying any initiated or neoplastic cells that might be formed.

The lack of increased Con A agglutination during the regenerative hyperplasia following freeze ulceration is in agreement with the lack of effect observed during the regenerative hyperplasia induced by the surgical insertion of glass beads as reported by Kakizoe et al. (11). Nevertheless, our observation that freeze ulceration followed by sodium saccharin, also a carcinogenic regimen (5), does not increase Con A agglutination suggests that this assay does not detect bladder carcinogenesis in all circumstances.

It is unclear at present what the mechanism is that leads to increased Con A agglutination after CP or after carcinogen administration. The effect appears rapidly after administration of these agents but disappears after cessation of chemical administration, even if tumors eventually appear (11). However, the amount of CP bound to the urothelial cells appears the same as in normal urothelium whether examined by lectin bound to fluorescein or to peroxidase (1, 3). Changes in the distribution of Con A receptors on the cell membrane rather than changes in the number of receptors are a possible explanation, but there currently is no evidence available to support this hypothesis.

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REFERENCES


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