Effect of N-Methyl-N-nitrosourea on the DNA of Rat Bladder Epithelium

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SUMMARY

Bladder cancer can be induced in the rat by the intravesicular administration of N-methyl-N-nitrosourea. DNA damage in rat bladder epithelial cells after administration of methyl nitrosourea has been examined by measuring the change in sedimentation of the DNA in alkaline sucrose gradients. A dose response of DNA damage in the urothelium was observed with single intravesicular doses of 0.1, 0.3, and 0.5 mg of methyl nitrosourea. Larger doses of methyl nitrosourea damaged the epithelium so extensively that biochemical studies were not feasible. DNA repair, measured by the return to a normal sedimentation pattern of DNA on alkaline sucrose gradients, was followed over a period of 9 days with the use of 0.5 mg of methyl nitrosourea to initiate the damage. Bladder epithelial cells were able to repair the DNA damage induced by methyl nitrosourea. However, the possibility of persistent damage not detectable by sedimentation of DNA on alkaline sucrose gradients cannot be ruled out.

INTRODUCTION

MNU\(^2\) is carcinogenic for a number of organs of several species, with the tissue specificity depending upon the route of administration and the dose schedule (summarized in Ref. 6; see also Refs. 16 and 20). Bladder cancer is produced in rats treated with multiple, intravesicular doses of MNU (4). More recently, Hicks et al. (5) have described a model system, using a single initiating but noncarcinogenic dose of MNU, to detect potential weak bladder carcinogens or cocarcinogens. The biochemical mechanism of action of MNU in this system is not known, although the toxic response of rat urothelium to a single intravesicular dose of MNU has been described in some detail (4, 22). Previous studies have shown that MNU interacts with nucleic acids in vivo (9, 12–14) and in vitro (7, 10, 11) to produce a variety of alkylated products. The liver, lung, kidney, and brain of the rat have the ability to repair the MNU-induced damage of DNA (1, 3, 14). However, data are not available concerning the response of rat urothelium to a single intravesicular dose of MNU. We have examined the damage and repair of DNA induced by MNU in the rat urothelium.

MATERIALS AND METHODS

Female Sprague-Dawley rats, weighing 120 to 140 g, were obtained from ARS Sprague-Dawley, Madison, Wis. MNU (Consolidated Midland Corp., Brewster, N.Y.) was dissolved in 0.9% NaCl solution at various concentrations immediately before use and administered by a urethral catheter in a volume of 0.15 to 0.2 ml. Animals were killed by decapitation. The bladders were quickly removed, everted on the end of a blunt 19-gauge needle, and rinsed in a solution of 24 mM EDTA-75 mM NaCl, pH 7.5. While still on the needle, the bladders were ligated at the neck and inflated with 0.3 ml of the EDTA-NaCl solution. The epithelial lining of the bladder was removed by placing the bladder in 0.3 ml of a lysis solution containing 0.3 M NaCl-0.03 M EDTA-0.1 M Tris-HCl buffer and 0.5% sodium dodecyl sulfate, pH 12, for 1 min. Aliquots of the lysed samples containing less than 5 µg of DNA were carefully layered on top of a linear 5 to 20% alkaline sucrose gradient prepared as previously described (2). All gradients were centrifuged for 1 hr at 25,000 rpm at 20° with the Beckman SW 40 rotor in a Spinco Model L2-65B ultracentrifuge. At the end of the run, the rotor was allowed to decelerate without the brake.

DNA sedimentation profiles were determined by a fluorometric procedure similar to that recently described by Zubroff and Sarma (23). After centrifugation, 16 fractions of 20 drops each were collected from the gradients into conical 5-ml centrifuge tubes. DNA was precipitated by placing the tubes in ice and adding the following in order with mixing after each addition: (a) 100 µl of 0.2% bovine serum albumin; (b) 20 µl of 4 N HCl; and (c) 500 µl of 40% trichloroacetic acid. The DNA was left to precipitate overnight at 0°. To remove the high concentrations of sucrose in the samples, the precipitated DNA was washed thoroughly as follows: (a) 3 times with 1 ml of cold 5% trichloroacetic acid, (b) once with 0.5 ml of 0.1 M potassium acetate in ethanol (allowed to sit in ice for 15 min), and (c) 2 times with 1 ml of ether. Following the last ether wash, the tubes were dried at least 2 hr. Between each wash, the tubes were centrifuged for 10 min at 2500 rpm in an International PR-2 centrifuge. To the dried samples, 20 µl of 3.5-diaminobenzoic acid (400 mg/ml in water) were added, and the tubes were heated at 70° for 30 min (8). The tubes were brought to room temperature, 0.5 ml of 0.6 N perchloric acid was added, and the fluorescence was read on a Turner Model 111 fluorometer (23).

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\(^2\)The abbreviation used is: MNU, N-methyl-N-nitrosourea.
For histological examination, bladders were removed and either everted and distended with buffered EDTA solution before being placed in Bouin’s fixative (Figs. 1 and 2) or simply inflated with Bouin’s without having been everted (Figs. 3 to 7). Sections were cut at 6 to 8 μm and stained with hematoxylin and eosin.

RESULTS

In order to study the damage and repair of DNA in the bladder epithelium by the alkaline sucrose gradient method, 2 initial considerations were of paramount importance: (a) it was essential to develop a procedure for removing the DNA from the urothelium with minimum physical and enzymatic damage to the DNA; and (b) it was necessary to find a dose of MNU that would not cause extensive necrosis and denudation of the urothelium but would damage the DNA. Removal of DNA from the urothelium for sucrose gradient analysis was accomplished by placing the everted, inflated bladder into an alkaline solution of sodium dodecyl sulfate. The urothelium was stripped from the bladder and the cells were lysed, yielding a viscous solution. It was found that a 1-min exposure to the lysing solution at pH 12 completely removed the epithelial lining, leaving the basement membrane intact (Figs. 1 and 2). The urothelium could also be removed at neutral pH by allowing the everted bladders to stand in a similar lysing solution at pH 7.4 for 15 to 20 min.

With respect to the 2nd point, it was found that a single intravesicular dose of 1 to 2 mg of MNU resulted in necrosis and stripping of the urothelium from extensive areas of the bladder, as reported by Hicks and Wakefield (4, 22). We therefore selected a single intravesicular dose of 0.5 mg of MNU for further study. At 1 hr after administration of 0.5 mg of MNU, there were no demonstrable histological changes in the bladder. Within 4 hr, marked edema in the lamina propria was seen (Fig. 3), but there was no loss of the epithelium or hemorrhage and inflammation. Although most of the epithelium remained intact at 24 hr after administration of the MNU, focal areas of denudation and ulceration of the mucosa were observed at this time (Fig. 4). Histological changes (edema, inflammation, denudation, focal hyperplasia, and nuclear pleomorphism) appeared to be maximal at about 48 hr (Fig. 5), but these changes were not as severe as reported for larger doses of MNU (4, 22). By 3 days, changes were less pronounced (Fig. 6). With the dose of 0.5 mg MNU, the epithelium was never totally lost from large areas of the bladder and there was not any generalized hyperplastic response. For purposes of this study, we did not follow the histological changes beyond 2 weeks (Fig. 7), at which time the picture was not much different from that seen at 3 days, except for occasional mitoses observed at 2 weeks.

MNU was given by urethral catheter at varying dosages, and DNA of the bladder epithelium was examined 1 hr later by alkaline sucrose gradient centrifugation. The gradient profiles of a control and animals treated with MNU at doses of 0.1, 0.3, and 0.5 mg/bladder are shown in Chart 1. A definite dose response is seen, with 0.5 mg giving the maximum effect, as can be observed by the gradient method. At a dose of 0.3 mg, the DNA was dispersed throughout the gradient, while very little effect was observed with a dose of 0.1 mg.

Rats were given 0.5 mg of MNU by a urethral catheter and were sacrificed at 1, 4, 24, and 72 hr (Chart 2) and at 9 days. At 4 hr after administration of MNU, the maximum effect on DNA sedimentation profiles was observed. By 24 and 72 hr after instillation of the MNU, DNA fragmentation was not as extensive. The sedimentation profile at 24 hr was broader, suggesting that some cells were damaged more than others or some had recovered more than others. At 9 days, the sucrose gradient profile was identical to that of the controls.

The data presented in Charts 1 and 2 are results from single animals at each dose or time point. However, each of these experiments was repeated at least 2 additional times to verify the reproducibility of the results. Results similar to those shown in Charts 1 and 2 were obtained.

DISCUSSION

DNA damage and repair have been shown to take place in rat bladder epithelial cells in vivo. A dose-dependent fragmentation of bladder epithelial DNA occurred following exposure of these cells to the carcinogen MNU. Although the molecular weight distribution of the fragments produced was not determined in our studies, recent work by others with this same alkaline sucrose gradient technique in which...
the gradients were calibrated using DNA from T₄-, T₇-, and SV40-nicked circles (15, 18, 23) indicates that these fragments are in the range of 1 to 9 x 10⁶ daltons. The size distribution of the DNA sedimenting near the bottom of the alkaline gradients in the controls is not known, but it appears to be single stranded and exceeds 1 x 10⁶ daltons (17). DNA released and sedimented by this method has a sedimentation rate too large to be measured (2, 17).

The histological studies clearly demonstrate that there is no major loss of epithelium or extensive hyperplasia of the bladder epithelium induced by the single dose of 0.5 mg of MNU used in these studies. Therefore, the return of the gradient profiles toward the control gradient profiles is probably not due to the collection of undamaged cells or the synthesis of new DNA due to cell replication. The shift in sedimentation of DNA to the control region of the gradients is interpreted to be due to repair of the DNA damaged by the MNU.

In these studies, our primary aim was to show qualitatively that MNU induces DNA damage and repair synthesis in rat bladder epithelial cells. Limitations of our present methodology do not permit precise quantitative studies on the types of lesions induced by MNU because of the small amounts of DNA present in the rat bladder epithelium (about 10 μg total). Three predominant types of lesions may occur in DNA after exposure to MNU. These are alkylation (7, 9–14), depurination, and strand breaks, the latter 2 lesions probably arising as a consequence of alkylation (for review, see Ref. 19). In other tissues studied, 7-methylguanine is the primary alkylated product, but it does not seem to be biologically important (7, 9, 14), even though it might account for most of the depurination and strand breaks. Apurinic sites in DNA induced in this manner are capable of being enzymatically repaired in mammalian systems (21). Other alkylated products, especially those interfering with normal base pairing in DNA, such as O₆-methylguanine, are believed to be of major biological importance (7, 9, 14).

It will be of some interest to determine the types of lesions induced in bladder epithelial cells by MNU and to find whether there is preferential repair of the different types of damage or persistence of alkylated products, such as O₆-methylguanine. However, such studies must await the development of more sensitive analytical techniques for determination of low levels of alkylated bases in DNA.

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REFERENCES

Fig. 1. Normal rat bladder. The bladder was everted and distended with 0.3 ml of buffered EDTA solution before fixation. × 200.
Fig. 2. Removal of transitional epithelium of rat bladder. The bladder was everted and distended, as in Fig. 1, immersed in an alkaline solution of sodium dodecyl sulfate for 1 min (see "Materials and Methods"), then rinsed and fixed. Compare luminal surface with that in Fig. 1. × 200.
Fig. 3. Bladder of rat 4 hr after intravesicular instillation of 0.5 mg MNU. Edema with marked widening of the lamina propria. × 100.
Fig. 4. Ulcerated area in rat bladder 24 hr after single intravesicular dose of 0.5 mg MNU. × 200.
Fig. 5. Rat bladder 48 hr after MNU. Edema of lamina propria. Epithelium is intact despite slight cellular atypicalities. × 200.
Fig. 6. Rat bladder 72 hr after MNU. × 200.
Fig. 7. Rat bladder 2 weeks after MNU. × 200.
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