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DETECTION OF REPAIR OF CHEMICAL-INDUCED DNA DAMAGE IN VIVO BY THE NUCLEOID SEDIMENTATION ASSAY

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The nucleoid sedimentation assay was used to study chemical-induced DNA repair in vivo. Nucleoid bodies were prepared from liver and lung of mice at various times after i.p. treatment with 1-methyl-1-nitrosourea or 4-nitroquinoline-1-oxide. Both carcinogens induced a dose-dependent loss in negative DNA supercoiling in liver and lung. The rate of DNA repair of 1-methyl-1-nitrosourea was similar in liver and lung whereas 4-nitroquinoline-1-oxide-induced DNA damage was repaired faster in lung than in liver. Results obtained by the nucleoid sedimentation technique corresponded to measurements of DNA repair by unscheduled DNA synthesis. The nucleoid sedimentation assay should be a useful tool to examine in vivo repair of chemical-induced DNA lesions in various tissues of laboratory animals.

Cells have developed mechanisms to repair chemical- or radiation-induced lesions in their DNA. DNA repair has been examined in great detail in mammalian cell culture systems (1, 2). In contrast, there have only been a few attempts to study repair of chemical-induced DNA damage in vivo, in part, because of the complexity to adapt the in vitro techniques to laboratory animals. The nucleoid sedimentation technique is a sensitive system to measure DNA repair in eucaryotic cells in culture (3-7). Nucleoid bodies are formed when cells are exposed to nonionic detergents and high salt concentration. They are nuclei depleted of most proteins and lipids. The sedimentation rate of nucleoids in sucrose gradients is dependent on the degree of DNA supercoiling. Introduction of only a few strand breaks in DNA can produce a detectable loss in negative DNA supercoiling (8, 9). In addition to its sensitivity, the nucleoid sedimentation assay has several other advantages to study DNA repair in vivo. Only a small number of cells are required per assay, and experiments can be performed without using radiolabeled compounds. Furthermore, the ease of the technique may

The abbreviations used are: EB, ethidium bromide; MNU, 1-methyl-1-nitrosourea; 4NQO, 4-nitroquinoline-1-oxide; UDS, unscheduled DNA synthesis.

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allow the simultaneous examination of repair in several tissues or cell types. Although repair was not investigated, Lipetz et al. (10) used the nucleoid sedimentation method to detect aflatoxin B_1-induced damage to rat liver DNA in vivo. In this report we first examine whether the nucleoid sedimentation assay can be used to study the repair of MNU- and 4NQO-induced damage to DNA in liver and lung of mice.

MATERIALS AND METHODS

Female A/HeJ mice (eight to ten weeks old) were treated either with MNU or 4NQO (Sigma Chemical Co., St. Louis, Mo.). MNU, in the dose range between 0.01 and 0.4 mmol/kg, was dissolved in 5 mM sodium acetate, pH 5, containing 0.15 M NaCl. Mice were injected with a single i.p. dose of 0.1 ml of the solution above or vehicle only (controls). 4NQO was given in DMSO:saline (0.87% NaCl) 9:1. Each mouse received a single i.p. dose (0.26 mmol/kg) of 50 µl.

At various times after administration with carcinogen or vehicle animals were sacrificed, liver and lung removed and kept in an ice cold solution containing 0.3 M sucrose, 10 mM Tris, 10 mM EDTA, pH 7.4. To prepare nucleoid bodies from liver and lung, whole organs were gently homogenized in a glass tissue grinder containing a solution of 10 mM 2-mercaptoethanol, 0.34 M sucrose, 60 mM NaCl, 15 mM Tris-HCl and 10 mM EDTA, pH 7.4. Livers were homogenized in 3 ml of the solution above using a rubber pestle. For lungs, a Teflon pestle and only 1.5 ml solution were used. Homogenates were filtered through a 125 µm mesh screen (Nitex, Namsco Inc., Stafford, Tx.). Nucleoids were then formed employing a modified procedure used by Cook and Brazell (5) in their work with cultured cells. Continuous gradients containing 2 M NaCl, 10 mM Tris, 10 mM EDTA, pH 8, and either 15-30% (w/w) sucrose (ultrapure: nuclease, protease, none detected, Bethesda Research Labs. Inc.) for liver and 12.5-25% (w/w) sucrose for lung samples were formed in ultraclear centrifuge tubes (5 ml) using a gradient maker (Buchler Instruments, Fort Lee, N.J.). To detect the nucleoid band the gradient solution also contained 1 µg/ml of the DNA-dye Hoechst 33258 (Calbiochem-Behring Corp., La Jolla, Ca.). The dye at this concentration does not influence the sedimentation of nucleoids (11). A lysis solution (2 M NaCl, 10 mM Tris, 10 mM EDTA, 0.5% (v/v) Triton X-100, pH 8) was then layered carefully on the top of the preformed gradient solution followed by 50 µl (4 to 8 x 10^5 cells) of the homogenized liver or lung sample. Tubes were then placed in precooled (4°C) buckets of a swinging out rotor SW 50.1 (Beckman). After 20 min of lysis time, gradients were spun in an ultracentrifuge L5-50B (Beckman) for 30 min at 4°C using a speed of 25,000 rpm for liver and 40,000 rpm for lung samples. The absolute sedimentation distance of the nucleoid band from the top of the tube was determined by detection of the visible fluorescence of the DNA-Hoechst dye complex using a longwave UV-lamp (Black-ray, 366 nm, Fisher Scientific, Pittsburg, Pa.). The sedimentation distance is calculated as the average distance of the top and the bottom of the nucleoid band.

RESULTS

To show that nucleoid bodies sediment as a function of their DNA supercoiling, they can be exposed to gradients containing various concentrations of the intercalating DNA dye EB. Nucleoids prepared from cultured cells sediment in a biphasic manner varying the concentration of EB, e.g., they show a reduced sedimentation distance (partial loss of negative DNA supercoiling) at low EB concentrations and a longer sedimentation distance (introduction of positive supercoiling) at higher EB levels (8). Nucleoid

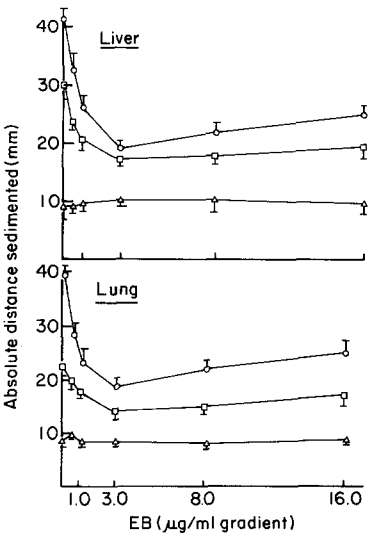


Figure 1. Exposure of nucleoids to various concentrations of EB. Nucleoids were prepared from liver and lung of mice 1 hr after treatment with an i.p. dose of MNU or vehicle and then were sedimented in a sucrose gradient containing various concentrations of EB. The absolute sedimentation distance (mm) of nucleoids from the top of the tube is plotted as a function of EB concentration in the gradient. (○) control; (□) 0.08 mmol MNU/kg; (△) 0.4 mmol MNU/kg. Each point represents the average ± S.D. of four experiments (control) and three experiments (MNU treatment).

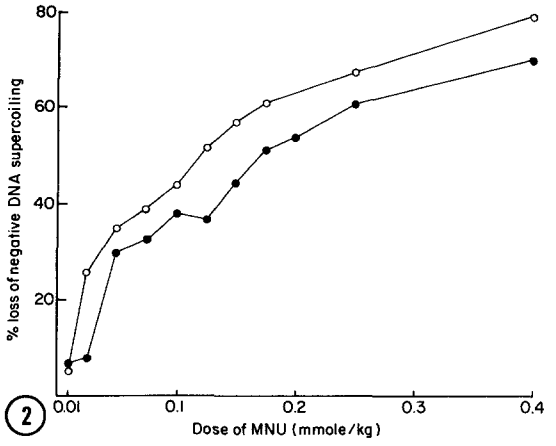
bodies prepared from freshly isolated liver and lung of control mice also showed the biphasic response to EB (Fig. 1). These results suggest that nucleoids obtained from whole organs have the same integrity as those prepared from cultured cells.

When cells are irradiated with a high dose of UV, the sedimentation of nucleoids is strongly reduced, and the curve obtained does not show the biphasic behavior in presence of EB because of the high extend of UV-induced DNA strand breaks formed during DNA repair (5). To examine the effect of DNA-damaging chemicals on the sedimentation behavior of nucleoids, we treated mice with a single i.p. dose of 0.4 or 0.08 mmol/kg of the direct methylating carcinogen MNU and then exposed nucleoids prepared from liver and lung to various concentrations of EB. Both doses of MNU induced a dose-dependent reduction in the sedimentation of nucleoids in absence of EB (Fig. 1). Nucleoid bodies prepared from mice treated with 0.4 mmol MNU/kg were insensitive to EB and thus behaved similar to nucleoids from cells irradiated with a high dose of UV. This suggests that DNA was extensively broken and in its most relaxed form (maximal reduced sedimentation distance). However, at the lower MNU dose of 0.08 mmol/kg, DNA was not fully relaxed and still contained enough intact regions to allow a further loss in negative DNA super-

coiling at low concentrations of EB and also introduction of a slight positive supercoiling at higher EB levels.

To evaluate the sensitivity of our system, we examined the dose-response relationship for MNU-induced changes in DNA supercoiling. Mice were treated with i.p. doses of MNU ranging from 0.01 to 0.4 mmol/kg. A reduction of 5% to 35% in negative DNA supercoiling was observed in nucleoids from liver and lung in the dose range between 0.01 and 0.05 mmol MNU/kg and a reduction of 70% to 80% at the higher dose of 0.4 mmol/kg (Fig. 2). A small increase in the MNU dose produced a greater loss in negative DNA supercoiling in the low dose range than at higher doses of the chemical.

Based on our results obtained from the dose response relationship, we chose a high (0.4 mmol/kg) and a low (0.08 mmol/kg) dose of MNU to study DNA repair. Restoration of nucleoid sedimentation to control level was dependent on the initial dose of carcinogen given to the animals (Fig. 3). The sedimentation distance of nucleoids prepared from liver and lung of



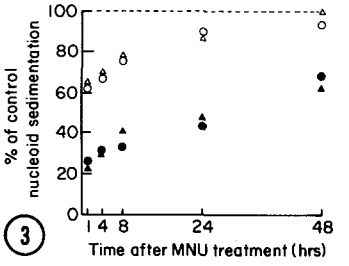


Figure 2. Dose response for MNU-induced loss of negative DNA supercoiling. Nucleoids were prepared from liver and lung of mice 1 hr after treatment with various i.p. doses of MNU or vehicle. Nucleoids were sedimented in a sucrose gradient and the absolute sedimentation distance of nucleoids from the top of the tube was determined. The % loss of negative DNA supercoiling is defined as (1-sedimentation distance of MNU nucleoids/sedimentation distance of control nucleoids) x100. (○) liver; (●) lung. Each point represents the average of two typical experiments.

Figure 3. Repair of MNU-induced DNA damage. Nucleoids were prepared from liver and lung of mice at various times after treatment with a single i.p. dose of MNU or vehicle. Nucleoids were sedimented in a sucrose gradient and the absolute sedimentation distance of nucleoids from the top of the tube was determined. The sedimentation distance of MNU nucleoids as % of control nucleoid sedimentation is plotted as a function of time after MNU treatment. Circles, liver; triangles, lung; open, 0.08 mmol MNU/kg; closed, 0.4 mmol MNU/kg. Each point represents the average of two typical experiments.

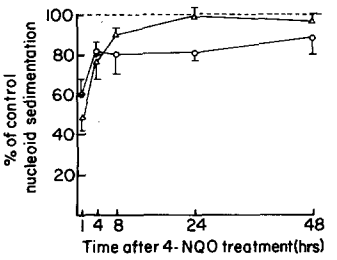


Figure 4. Repair of 4NQO-induced DNA damage. Nucleoids were prepared from liver and lung of mice at various times after treatment with vehicle or a single i.p. dose of 0.26 mmol 4NQO/kg. Nucleoids were sedimented in a sucrose gradient and the absolute sedimentation distance of nucleoids expressed as % of control nucleoid sedimentation distance is plotted as a function of time after 4NQO treatment. (○) liver; (△) lung. Each point represents the average ± S.D. of four separate experiments.

mice treated with 0.08 mmol MNU/kg returned to control values 48 hr after treatment with carcinogen. In contrast, a significant reduction (30% to 40%) in sedimentation distance was still present 48 hr after treatment with the higher dose of 0.4 mmol/kg of the chemical. The time course of MNU-induced repair was similar in liver and lung at both doses examined.

We also investigated a DNA damaging agent which must be metabolically activated to interact with DNA. The lung carcinogen 4NQO was chosen since it induces UDS *in vivo* in lung and liver of A/HeJ mice (12). The chemical produced a loss of negative DNA supercoiling of 40% in liver and 50% in lung nucleoids, respectively, 1 hr after a single i.p. dose of 0.26 mmol/kg (Fig. 4). 4NQO-induced damage to DNA was repaired rapidly in lung. In the time period from 1 to 8 hr posttreatment, the nucleoid sedimentation rate went from 50% to 90% of control and reached control value 24 hr after treatment with the chemical. In contrast, the repair of 4NQO-induced DNA damage was slower in liver than in lung. The sedimentation value in liver had not returned to control value 24 hr after treatment with 4NQO.

DISCUSSION

This is the first report using the nucleoid sedimentation assay to examine *in vivo* repair of chemical-induced DNA damage in various tissues of laboratory animals. The system was able to detect 4NQO- and MNU-induced damage to DNA and to examine the repair of this damage in liver and lung. We chose MNU and 4NQO in this study to compare repair data examined by the nucleoid sedimentation technique with results obtained by UDS. Kaufmann *et al.* (12) investigated repair in rat liver after a single i.p. dose of 0.25 mmol MNU/kg. The repair curve they obtained from UDS measurements was similar to the one we obtained by the nucleoid sedimentation method

(Fig. 3). Both types of measurements showed that repair was initially rapid and then proceeded at a slower rate. Also, the dose-response curve for MNU-induced changes in nucleoid sedimentation in liver of mice (Fig. 2) had the same shape as the dose-response curve for MNU-induced UDS in rat liver (12). Furthermore, Kulkarni and Anderson (13) demonstrated that 4NQO induced UDS in lung of mice 2 hr after a single s.c. dose of 0.5 mmol/kg. We have also shown that 4NQO induced UDS in both lung and liver after a single i.p. dose of 0.26 mmol/kg, the same treatment used in this report (unpublished observation). The repair curve obtained from measurements of UDS is identical to the repair curve in Fig. 4 from the nucleoid sedimentation technique. It is satisfying that similar conclusions are reached from the two types of measurements of repair of MNU- and 4NQO-induced DNA damage. These results suggest that the nucleoid sedimentation assay represents another method which can be employed to study in vivo repair of chemical-induced damage to DNA.

The degree of DNA supercoiling and thus, the sedimentation rate of nucleoids, is very sensitive to strand breaks in DNA (5). Strand breaks are produced during excision repair of damaged bases. The general agreement of the repair data obtained by the nucleoid sedimentation assay and UDS is probably a reflection that nucleoid sedimentation reveals the influence of single strand breaks generated during excision repair, whereas UDS measures the incorporation of radiolabeled bases into newly synthesized repair patches. However, it should be noted that treatment of animals with chemicals in vivo can produce DNA strand breaks by processes other than excision repair of damaged bases. Such mechanisms could include 1) formation of phosphotriesters, 2) destabilizing effect on DNA structure due to carcinogen-DNA adducts and 3) formation of carcinogen-induced hydroxyl-radicals. In addition, loss of negative DNA supercoiling can occur without breaking DNA. The mere presence of bulky carcinogen-adducts may destabilize the tertiary and quarternary structure in such a way to cause a loss in DNA negative supercoiling as seen in SV40 viruses incubated with aflatoxin B_1 or benzo(a)pyrene (14). Whether or not adduct levels are high enough in vivo to cause a loss in negative DNA supercoiling without producing strand breaks is unknown.

There have been several methods employed to measure chemically-induced DNA damage and repair in vivo. Eastman and Bresnick (15) adapted the alkaline elution technique developed by Kohn et al. (16) to detect breakage and repair of DNA alkylated in vivo. Furthermore, excision repair of various carcinogen DNA adducts was investigated by measuring the incorporation of radiolabeled thymidine into newly synthesized repair patches (UDS) either directly by isopycnic gradients of isolated DNA (13, 17) or by autoradiography (18). Stewart (19) developed a benzoylated DEAE-cellulose

chromatography technique to examine carcinogen-induced repair intermediates in rat liver. The nucleoid sedimentation assay has several advantages for in vivo studies of DNA repair. First, no radioactive compounds are needed and only a small number of cells are required. In addition, the relative ease and rapidity of the technique allows simultaneous repair studies in several tissues or cell types. In this report we developed the assay for liver and lung of mice but the system is adaptable to other tissues and cell types. In conclusion, we believe the nucleoid sedimentation technique will be a useful tool to investigate repair of chemical-induced DNA lesions in a variety of tissue and cell types of laboratory animals.

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