Patterns of Damage and Repair of Liver DNA Induced by Carcinogenic Methylating Agents in Vivo

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SUMMARY

Single-strand breaks induced by methylating agents in liver DNA and their joining were studied in vivo by the use of alkaline sucrose gradients. DNA breaks were induced by the i.p. injection of single doses of dimethylnitrosamine (DMN), methylazoxymethanol acetate (MAM), N-methyl-N-nitrosourea (MNU), and N-methyl-N-nitrosourethan. The degree of damage was dependent on the dose of the substance given. DMN and MAM are comparably efficient in inducing marked single-strand breaks of liver DNA in small doses. MNU, although noncarcinogenic for liver, causes single-strand breaks of liver DNA, while N-methyl-N-nitrosourethan causes less damage even at toxic doses. The repair of the damage to DNA induced by MNU was complete in the first week while that induced by DMN and MAM was still not complete within 14 days after administration of the compound. Thus, the repair of the damage induced by hepatocarcinogens (DMN and MAM) seemed to be slower than that with the methylating agents not carcinogenic for the liver (MNU and methyl methanesulfonate).

INTRODUCTION

Chemical carcinogens, or their active metabolic products, have been found to interact covalently with all the major groups of macromolecules in target cells including protein, RNA, and DNA (2, 18, 28–30, 34). Although these reactions are interesting, their relation to cellular neoplasia is not understood (11, 12).

The function of DNA as a dynamic repository of information and the importance of this macromolecule in so many aspects of the function of normal cells makes DNA an important focus for study. Understanding the nature of carcinogen produced tissue DNA damage and its repair may be an important aspect in the molecular analysis of carcinogenesis. The recent work on DNA damage and repair in cells from patients with xeroderma pigmentosum (4, 5) warrant an increased emphasis on the study of DNA damage and repair in carcinogenesis.

This report is the 2nd of a series of studies the objective of which is to understand, in increasing detail, the patterns, nature, and metabolic control of the damage and repair of DNA as a consequence of the action of selected carcinogens on tissues in which cancer develops. The initial approach is to use groups of carcinogens with some common features, such as ability to methylate or ethylate DNA or to react at certain sites with components of DNA. Hopefully, certain patterns may be uncovered in which 1 or more specific interactions may be correlated with certain kinds of damage and repair.

In this report, a number of carcinogens, all of which are known to methylate DNA in 1 or more sites, are examined. Some are carcinogenic for the liver and others are not (3, 9, 24, 26, 27, 34, 38). Although this study is young, certain suggestions relating patterns of repair with carcinogenicity may be made.

MATERIALS AND METHODS

Animals used in these experiments were white male Wis-tar rats (Carworth Farms, Rockland County, N. Y.) weighing approximately 100 g. All animals were partially hepatectomized according to the procedure of Higgins and Anderson (13), and liver DNA was labeled with thymidine-methyl-3H (New England Nuclear, Boston, Mass.) during the period of regeneration, as described in the preceding paper (6).

DMN* (K and K Laboratories, Plainview, N. J.), MNU, MNU, and MAM (Schwarz/Mann, Orangeburg, N. Y.) were dissolved or diluted with 0.9% NaCl solution and injected i.p. at varying dosage and time intervals, depending on the experiment. Animals were killed by decapitation, and liver cell suspensions were prepared in 0.024 M EDTA-0.075 M NaCl, pH 7.5, using squid technique as previously described (6). The preparation of alkaline sucrose gradients, lysing of cells on the gradient, and other technical details were performed as given in the preceding

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Methylating Agents and Liver DNA Damage

The value of the hepatic DNA from rats treated with DMN (10 \( \mu \)g/g body weight) for 4 hr was calculated using the method of Abelson and Thomas (1). T\(_4\) phage DNA was used as the marker.

RESULTS

Each gradient represents 1 animal. Each experiment was repeated a minimum of 3 to 4 times. Similar patterns of results were obtained. On each gradient not more than 1 \( \mu \)g of DNA (300 to 700 cpm) was layered.

Time Course of Induction of Single-Strand Breaks by DMN. DMN was injected i.p. and the rats were killed 15 min and 1, 2, and 4 hr after injection. By about 15 min after injection, the amount of heavy DNA sedimenting normally in the 1st 3 tubes was significantly decreased and some lighter DNA, as represented by a shift of the radioactivity peak and trailing of radioactivity into the midportion of the gradient, began to appear (Chart 1). After 1 hr, there was very little of the intact heavy DNA and the majority of DNA formed a peak in the midportion of the gradient. After 4 hr the DNA was mostly in small pieces which sedimented in the upper part of the gradient as a large peak.

This peak remained unchanged for up to 12 hr. From these data, it is evident that DMN injected i.p. in the dosage used reacts very quickly and causes an almost immediate change in the liver DNA as monitored by single-strand breaks in an alkaline medium. The maximum effect is obtained after 4 hr. The acid-precipitable radioactive peak from a 4-hr DMN-treated DNA sample was analyzed to determine the S value. For this purpose, the sample was spun at 25,000 rpm but to different lengths of time. The DNA pieces were heterogenous and had a S value ranging from 76 to 129 (S. E. Abanobi and D. S. R. Sarma, unpublished data). T\(_4\) phage DNA was used as marker and, under identical centrifugation conditions, the S value of T\(_4\) phage DNA was determined using the method of Abelson and Thomas (1). T\(_4\) phage DNA had a S value of 62.

Dose Response to DMN, MAM, MNU, and MNUT. In the experiments to be described, the animals were killed 4 hr after the administration of the methylating agent. DMN in a dose of 1 mg/kg induced a slight shift of the sedimentation pattern of liver DNA on alkaline sucrose gradients (Chart 2). Doses below 1 mg/kg did not cause any detectable changes in the sedimentation pattern of liver DNA. A dose of 5 mg/kg caused more liver DNA single-strand breaks than the smallest effective dose, and the DNA sedimented over a broad range (Chart 2). Damage to all the DNA was achieved with a dose of 10 mg/kg. This dose of DMN caused a complete shift of the sedimentation pattern of liver DNA which appeared near the top of the gradient.

The lowest 4-hr effective dose of MAM was 1 mg/kg which caused a decrease in the radioactivity in the normal position and spreading of the peak (Chart 3). The shift with the 10 mg/kg was intermediate between that seen with 1 and...
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MNU, as the effect of MNUT was not comparable to that observed with the other agents. The lowest dose of a given methyediting agent causing a substantial but comparable shift of DNA on the alkaline sucrose gradient was used.

Chart 4. Effect of MNU on the sedimentation of hepatic DNA in alkaline sucrose gradients. MNU was administered i.p. at 40 (●), 80 (○), and 160 (★) mg/kg body weight. The rats were killed 4 hr after the administration of MNU or equivalent volume of 0.9% NaCl solution. Lysis of the liver cells and centrifugation of the gradients were the same as in Chart 1. Arrow, position on the gradient where the liver DNA of a control rat would band.

Chart 5. Effect of MNUT on the sedimentation of liver DNA in alkaline sucrose gradients. MNUT was given i.p. at 50 (●), 150 (○), and 200 (★) mg/kg body weight. The rats were killed 4 hr after the administration of MNUT or equivalent volume of 0.9% NaCl solution. Lysis of the liver cells and centrifugation of the gradients were the same as in Chart 1. Arrow, position on the gradient where the liver DNA of a control rat would band.

Chart 3. Effect of MAM on the sedimentation of liver DNA in alkaline sucrose gradients. MAM was given i.p. at 1 (★), 10 (●), and 25 (○) mg/kg body weight. The rats were killed 4 hr after the administration of MAM or equivalent volume of 0.9% NaCl solution. Lysis of the cells and centrifugation of the gradients were the same as in Chart 1. Arrow, position on the gradient where the liver DNA of a control rat would band.

25 mg/kg. A maximum effect, as judged by the shift toward lighter density of the radioactive peak on the alkaline sucrose gradient, was obtained with a dose of 25 mg/kg. Higher doses did not exert any further effect on the sedimentation pattern of liver DNA at 4 hr.

MNU was tested in doses ranging from 20 to 160 mg/kg. The lowest effective dose of MNU was 40 mg/kg (Chart 4). With a dose of 80 mg/kg, an amount used in carcinogenesis studies with a single administration (23), a shift of the peak of radioactivity toward lighter density comparable to the one obtained with DMN (10 mg/kg) was induced. The maximum effect was obtained with a sublethal dose of MNU (160 mg/kg).

Even rather high doses of MNUT (up to 200 mg/kg) injected i.p. did not cause any substantial shift of the bulk of liver DNA (Chart 5). With increasing amounts of the compound injected, there was nevertheless more DNA sedimenting in the midportion of the gradient, but a complete shift of the main peak of radioactivity toward the top of the gradient could not be achieved with nonlethal doses of MNUT at 4 hr following the administration of MNUT.

Repair of DNA Damage Induced by DMN, MAM, and MNU. The above shifts of the radioactive peak toward the lighter side on the alkaline sucrose gradients were interpreted as being due to DNA single-strand breaks (6). To observe the repair of DNA damage, liver DNA from animals treated with methylating agents was studied on alkaline sucrose gradients at various longer intervals after the administration of the chemical. The time-course experiments were limited to the study of DMN, MAM, and
Twenty-four hr after the injection of DMN at a dose of 10 mg/kg, the sedimentation pattern showed 2 peaks (Chart 6). Approximately 40% of total DNA sedimented in the lowermost part of the gradient corresponding to the sedimentation pattern of the intact heavy DNA. The remainder of DNA formed a lighter peak in the upper part of the gradient. This pattern did not markedly change during the next 24 hr.

The sedimentation patterns of the DNA damaged by MAM (25 mg/kg) showed a single peak at both 24 and 48 hr after injection, but the peak at 48 hr was broader and had spread toward the bottom of the gradient (Chart 7). At 24 hr there was a shift of DNA peaks towards the heavier side. At 48 hr the sedimentation pattern indicated that some of the DNA was repaired, but the major portion was still in the form of lighter fragments.

The patterns of repair of DNA damage, induced by MNU (80 mg/kg) at 24 and 48 hr, are illustrated in Chart 8. At 24 hr there was a slight shift of DNA peak towards the heavier side. The repair pattern at 48 hr showed that some of the DNA corresponded to the DNA of the controls.

The damage induced by all the 3 alkylating agents was not completely repaired in the 1st 48 hr after the administration. This is in contrast to MMS where repair was apparently completed by this time (6). In order to follow the patterns of repair, alkaline sucrose gradient analysis was performed at 6 and 14 days after the initial damage.

Repair after 6 and 14 Days. Six days after the i.p. administration of DMN, MAM, and MNU, only the damage induced by MNU was almost completely repaired (Chart 9). The sedimentation patterns of liver DNA obtained from DMN- and MAM-treated animals indicated that a large portion of the DNA sedimented near the bottom of the gradient, presumably repaired. Nevertheless, there was a considerable amount of DNA that sedimented toward the midportion of the gradient.

Analysis of sedimentation patterns at 14 days indicated that MNU-induced damage remained completely repaired, i.e., repair seen at 6 days was stable up to at least 14 days (Chart 10). DMN-induced damage was almost completely repaired with only about 12% of total
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described above clearly showed that the damage induced by various methylating agents was not repaired with equal facility. Therefore, an experiment was done to test whether the lesser damage induced by a smaller dose is repaired more readily and whether a "more extensive" damage requires longer time for repair.

DMN was given in 3 doses, 5, 10, or 20 mg/kg, and the sedimentation pattern of the DNA was measured after 6 days. Chart 11 shows that the damage induced with 5 mg/kg was completely repaired by this time. The repair was only partial with the 10-mg/kg dose and somewhat less with the highest dose (20 mg/kg) of DMN administered.

**Histological Changes in the Liver.** Fundamental to any study of DNA damage and repair is the question of cell integrity and survival. DMN and MAM are carcinogens that can induce severe liver cell death and necrosis in higher doses. Careful histological study was made of the liver of all animals at all time intervals and at all doses after the administration of each of the methylating agents. DMN at 5 and 10 mg/kg gave no indication by light microscopy of any irreversible cell damage and necrosis at time intervals of 4 hr to 14 days. MAM at 25 mg/kg and MNU at 80 or 160 mg/kg produced no observable necrosis of any liver cells at time intervals from 4 hr to 14 days. MNUT at doses of 150, 200, and 250 mg/kg likewise induced no liver cell damage. Thus, with all 4 compounds and at the dosages used, the detected DNA changes cannot be attributed to obvious loss of cell viability and the subsequent changes associated with necrosis. Presumably, all the changes were taking place in viable hepatocytes or other liver cells and can be considered as some form of molecular repair in an intact cell, rather than extensive breakdown of the DNA in a dying or dead cell.

DNA sedimenting in the midportion of the gradient as 2 small peaks. However, after 14 days, DNA from animals treated with MAM had not returned to the control pattern, indicating that complete repair did not occur.

**Dose-dependent Span.** The data from the experiments...
DISCUSSION

It is evident that all 4 methylating agents used in this study, as well as MMS (6), induce alterations in liver DNA in vivo which can be monitored as single-strand breaks by the use of centrifugation in an alkaline sucrose gradient. With this method and with most of the dosages used, the damage to DNA is apparently maximal within a few hr after the administration of each of the 5 methylating agents. The breakdown of DNA accompanying necrosis itself introduces uninterpretable complexities in this type of analysis and must be avoided.

The single-strand breaks observed with the methylating agents may not exist as such in situ but may be induced by the alkaline treatment used to separate the 2 strands of DNA. 7-Methylguanine is a major product of the action of most methylating agents on DNA (7, 17, 23, 31, 35) including liver DNA (37). This base, when present in nucleic acids, especially in DNA, makes the molecule very susceptible to depurination at that site in alkaline solutions and may result in strand break (18, 19, 25). Thus, the use of alkali in the analysis may overestimate the amount of single-strand damage existing in situ.

The patterns of repair of single-strand damage of DNA also vary with the methylating agent used. For example, with MMS (6), the damage is apparently repaired fairly rapidly, within 48 hr. However, with MNU, which produces a shift in DNA size equal to that with the doses of DMN or MAM used, repair was essentially complete between 48 hr and 6 days and remained so at 14 days. In contrast, with DMN and MAM, the repair process was delayed. With these latter agents, considerable damage was evident at 6 days and with MAM was not completely repaired at 14 days. Thus, with 2 potent hepatic carcinogens, MAM and DMN, the repair of single-strand damage is unusually slow. DMN (8) and the β-glucoside of MAM, cycasin (14), have each been reported to induce liver cancer in rats by a single administration under some experimental conditions. In contrast, MNU and MMS have thus far not been shown to be carcinogenic for the liver even though they do induce neoplasms in other organs (3, 9, 24, 27, 34, 38). However, this suggestive correlation between carcinogenicity and delayed repair must be examined with many more agents and under more varied experimental conditions in order to test its validity.

One feature of the repair process that seems to be valid with all 3 of the compounds producing maximal damage is its apparent dual nature. One part of the DNA appears to be relatively rapidly repaired, within about 48 hr, while the remainder of the DNA is much more slowly converted to longer strands. With MNU, the repair is complete within about 6 days while with DMN and MAM it is considerably slower. A similar phenomenon, conceivably analogous to the findings in vivo, is the apparent dual nature of the repair process in cells in vitro (10, 32). Whether the analogy is more than superficial must await detailed knowledge about enzymatic mechanisms in each case. Also, whether these differences in the rate of repair at early and later times are a reflection of different chemical (20–22) or physical sites of methylation of DNA or of different cells repairing at different rates remains a very important question for study.

DMN appears to be more efficient on a concentration basis in inducing DNA damage than are MNU, MNUT, or MMS. It is now clearly established that DMN and other nitrosamines require metabolic conversion to an active methylating agent (26). Thus, the active form is generated in situ and is apparently highly efficient under such circumstances. With MNU, MNUT, and MMS, each an active methylating agent per se, their stability must be such as to prevent their reaching the target DNA in concentrations comparable to that of the active form of DMN. MNUT probably decomposes in the body at such a rapid rate that sufficient amount probably does not reach the liver under the conditions used.

The dynamics of DNA damage and repair in vivo differ from that observed thus far in vitro. In vitro, proximate carcinogens and physical agents cause both single- and double-strand breaks of DNA rapidly (10, 15, 16, 33, 36). Using progressively higher doses of an agent, one can induce proportionately more breaks (15). Also in vivo, the induction of single-strand breaks in DNA under the conditions we have used is fairly rapid and the maximal effect is obtained within hours. The repair of the in vitro-induced damage is fast and occurs in a period of minutes or hours irrespective of the damaging agent (15, 16, 33, 36). The process is much slow in vivo and depends very much on the agent used. Thus, it appears that one cannot directly compare the data obtained in vitro and in vivo as the dynamics appear to be different. Also, since no differences were discovered in vitro between the damaging effects of carcinogenic and noncarcinogenic chemicals, the study in vivo may reflect more closely the actual events preceding the malignant transformation of cells in an intact organism.

It is evident that with the methods used in this study many interesting aspects of DNA damage and repair can be explored in the liver under a wide variety of conditions related to some aspects of carcinogenesis. Hopefully, new insights may be obtained from this and other approaches that will help to delineate in a clearly defined manner the possible importance of DNA damage and repair in carcinogenesis.

REFERENCES

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