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Evidence of Carcinogen-Induced Replication of Partially-Repaired DNA in Target Cells During Nitrosamine Carcinogenesis*

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Abstract—*Damage and repair of rat kidney DNA, following administration of the renal carcinogens dimethylnitrosamine and diethylnitrosamine, has been analysed by chromatography on benzoylated-DEAE-cellulose. For this purpose, DNA was labelled* in vivo *by administration of* ^{3}H-*thymidine* 30 hr *after injection of folic acid. After a recovery period of* 10 days, *single doses of nitrosamines were administered by i.p. injection. Both compounds caused a dose-dependent increase in the proportion of renal DNA bound to benzoylated-DEAE-cellulose due to the presence of single-stranded regions. In the case of dimethylnitrosamine, greatest structural damage of DNA was detected after completion of the alkylation reaction* in vivo. *The recovery of renal DNA from nitrosamine-induced damage was monitored in a sequential study. With dimethylnitrosamine, a biphasic pattern was recorded, maximal damage to DNA being evident* 1 *and* 4 days *after administration of the carcinogen. The increase at* 4 days *may be attributed to dimethylnitrosamine-induced proliferative activity in the kidney. The data are discussed with reference to the role of replication of cells containing persistent carcinogen-induced damage in this tumour induction system.*

INTRODUCTION

UPON administration to rats the carcinogens dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) are metabolised in the liver, and to a lesser extent in the kidney and other organs, with the production of an alkylating intermediate which may react with nucleic acids [1]. Elimination of the various alkylated bases from hepatic DNA *in vivo* following a single dose of DMN is accompanied by structural change in the macromolecule as evidenced by an increase in the proportion of DNA bound to benzoylated-DEAE-cellulose (BD-cellulose) columns [2, 3]. This binding has been interpreted in terms of the production of single-stranded regions in DNA [4, 5]. Further analysis has established that such single-stranded regions are associated with the repair of O^6-methylguanine rather than of 7-methylguanine [6]. Akthough alterations in the macromolecular structure of DNA may be detected over a period of several days following the administration of the carcinogen [7], the operation of DMN-induced repair processes in rat liver following a single dose may be deemed successful to the extent that, under normal conditions, administration of a single dose of DMN does not cause liver tumours [8].

The production of kidney tumours by a single dose of DMN has been correlated with the persistence in this target tissue of O^6-methylguanine in contrast to the relatively rapid elimination of this promutagenic base from hepatic DNA [9]. Organ-specific persistence of O^6-alkylguanine has been correlated with tumour site for a number of alkylating nitroso compounds [10–12]. Despite these correlations, a series of papers have appeared which suggest the persistence of O^6-alkylguanine is inadequate of itself to account for either the dose response or the target organ of these nitroso compounds [13–15]. On the other hand, induction of liver tumours by administration of a single dose of DMN within a certain period after partial hepatec-

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tomy suggests that proliferation of specific cell populations may play a critical role in nitrosamine carcinogenesis [16].

We have made a structural analysis, using BD-cellulose chromatography, of renal DNA following a single carcinogenic dose of DMN and also of DEN with a view to further clarifying the relationship between carcinogen-induced DNA damage and tumour induction. The data differ in certain respects from earlier observations obtained following analysis of liver DNA [7]. As a result tumour induction in this experimental system may be correlated with carcinogen-induced replication of a specific cell population superimposed upon, and augmenting, pre-existing structural damage to DNA.

MATERIALS AND METHODS

The materials and procedures used in this study have been described [7]. However, certain modifications were necessary to permit application of the methodology to analysis of renal DNA. For the purpose of chromatography, rat kidney DNA was radioactively labelled by administration of ^{3}H-thymidine after injection of folic acid. Female Wistar rats (200 g) were injected i.p. with 20 μCi ^{3}H-Me-thymidine (specific radioactivity 18.3 Ci/mmole, The Radiochemical Centre, Amersham, U.K.) 30 hr after intravenous injection of 250 mg/kg folic acid [17]. Preliminary experiments using these animals had indicated such treatment caused a 20-fold increase in the incorporation of ^{3}H-thymidine into renal DNA. After a recovery period of at least 10 days, DMN or DEN was administered by i.p. injection. Animals receiving DMN were dietary-conditioned to increase the level of renal metabolism of the nitrosamine [18] and ultimately the incidence of kidney tumours [19]. A mixture of glucose and sucrose was fed for 3 days before and 1 day after treatment with the carcinogen. DNA was invariably prepared from the pooled kidneys of identically-treated animals. The methods of extraction and chromatography on BD-cellulose were as previously described [7].

RESULTS

Administration of DEN caused an increase in the fraction of renal DNA bound to BD-cellulose in the presence of 1.0 M NaCl, and subsequently eluted with 1.8% caffeine-1.0 M NaCl solution. Although there was no alteration in the elution profile of renal DNA from BD-cellulose 4 hr after administration of 200 mg/kg DEN, by 24 hr the amount of caffeine-eluted DNA was increased above that in preparations of DNA from control animals. The increase was proportional to the dose of DEN administered (Fig. 1). A dose of 200 mg/kg DEN induced a 59% increase in the proportion of caffeine-eluted DNA. With increasing time, a slow recovery in the size of the caffeine-eluted fraction was observed, the proportion being 52% at 3 days and 41% by 5 days after DEN.

Isolated 4 or 24 hr after administration of DMN, the amount of caffeine-eluted renal DNA was approximately proportional to the dose (Fig. 1). However, the amount was

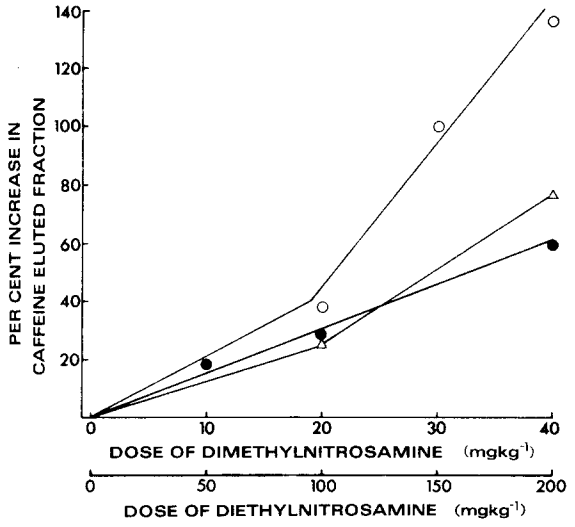

Fig. 1. The relationship between increased amount of caffeine-eluted renal DNA and the dose of DMN and DEN. Animals were killed 4 (△) or 24 (○) hr after DMN and 24 hr after DEN (●) injection as described in the Materials and Methods section.

approximately 50% greater at 24 hr than at 4 hr. Measurements using a 40 mg/kg dose indicated that the level of caffeine-eluted DNA remained approximately constant at 4, 8 and 12 hr after treatment indicating a sharp rise in the proportion of caffeine-eluted DNA during the second 12-hr period after treatment. Maintenance of animals treated with DMN on a glucose–sucrose diet markedly affected the proportion of caffeine-eluted renal DNA. In a single experiment in which DMN-treated animals were fed a normal diet a 17% increase in caffeine-eluted DNA was detected 4 hr after administration of 30 mg/kg: less than half that induced by 20 mg/kg in dietary conditioned animals.

Using elution from BD-cellulose as the basis of analysis, the time course of DMN-induced single-stranded regions in renal DNA following a 40 mg/kg dose was monitored over a period of 7 days. From a maximum level 24 hr after carcinogen treatment, the proportion of caffeine-eluted DNA fell rapidly (Fig. 2). Between days 2 and 3, the level decreased slightly. Four days after treatment there was a sharp increase to more than double the previous level. After the 5th day, the size of the caffeine-eluted fraction again decreased rapidly. By 7 days the proportion of caffeine-eluted DNA was approximately 25% greater than that from control animals, having decreased from a maximum of 140% greater recorded within 24 hr of administration of the carcinogen.

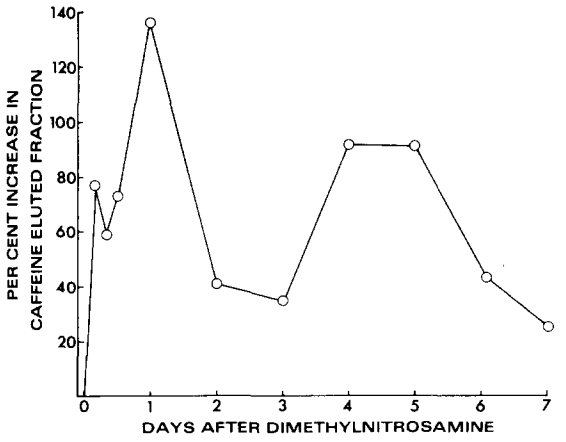

Fig. 2. Variation in the proportion of caffeine-eluted DNA in preparations isolated from kidneys of rats at various times after treatment with 40 mg/kg *DMN. The pooled kidneys of* 2 *animals were used for each experiment. Each point is the mean of at least* 2 *such experiments.*

DISCUSSION

Single doses of either DMN or DEN induce renal tumours in rats [19, 20]. Chromatography on BD-cellulose may be used to demonstrate production and repair of single-stranded regions in DNA of the target organ following administration of the renal carcinogens DMN and DEN. The largest increase in caffeine-eluted DNA induced by DEN (24 hr after 200 mg/kg) was less than half that from animals receiving a physiologically similar dose of DMN (40 mg/kg). This difference was almost certainly due to the efficacy of dietary conditioning [18, 19].

In contrast to extensive studies which have been made of the repair of DMN-derived methylated bases in renal DNA [9, 15, 21, 22], little has been reported concerning metabolism of ethylated bases following administration of DEN. This lack of supporting data concerning DEN dictates that most of the discussion be concerned with DMN. Detection 24 hr after treatment with DMN of a much increased amount of caffeine-eluted DNA relative to that at 4 hr contrasts sharply with an analogous study using hepatic DNA [7]. In livers, increase in single-stranded regions paralleled the methylation of DNA, both parameters being maximal 4 hr after a dose of 10 mg/kg DMN [7]. The methylation of nucleic acids *in vivo* by DMN is precisely correlated with the metabolism of the carcinogen [23, 24]. In kidney, therefore, a lag is evident between methylation, complete 4–12 hr after injection depending upon the dose [25, 26] and DNA damage, maximal at 24 hr (Figs. 1 and 2). Hence the increase in the fraction of renal DNA containing single stranded regions from the 4 to 12 hr level to that 24 hr after treatment (Fig. 2) suggests a difference in certain DNA repair processes between liver and kidney. Enzymic elimination of alkylated bases, including O^6-methylguanine, from DNA may be accomplished by a glycosylase-associated endonuclease and exonuclease activities followed by polymerase and ligase reactions to complete the repair process [27]. The present findings may be interpreted in terms of a more active exonuclease and preceding reactions on the one hand and a rate limiting polymerase on the other. Such disparity might account for progressive accumulation of single stranded regions in renal DNA during the first 24 hr after DMN treatment. A hypothesis concerning rapid nuclease activity at this time is compatible with loss of O^6-methylguanine from renal DNA at a rate comparable to that from liver [21]. Only after 18 hr is there almost complete cessation of this excision activity in kidney.

The complicated repair profile (Fig. 2) is in marked contrast to the regular pattern of progressive recovery observed in liver [7] after more extensive *in vivo* alkylation, respective levels of 7-methylguanine being 220 and 325 per 10^5 guanine [22, 26]. The conspicuous increase in amount of caffeine-eluted DNA from kidney 4–6 days after DMN is explicable in terms of DNA replication. Replicating DNA requires caffeine-elution from BD-cellulose (ref. 28 and B.W. Stewart, unpublished work). In both the epithelial and mesenchymal cells of the renal cortex, considered to be progenitors of DMN-induced kidney tumours, Hard reported a marked stimulation of proliferative activity initiated 3 and 4 days

respectively after DMN treatment [29]. The present data (Fig. 2) are derived principally from tubular epithelium of the cortex [30] though it seems probable that some resident interstitial cells were labelled by the folic acid treatment. The data, taken together with available autoradiographic [28] and biochemical [31] analysis of the kidney, strongly imply that the increase in caffeine-eluted DNA on days 4 and 5 is due to the presence of replicating DNA. Decrease in caffeine-eluted DNA during days 5–7 may be due to the operation of a post-replication, possibly error-prone, process [32].

The relevance of DNA structural change to induction by DMN of renal neoplasms may have been demonstrated by the "split dose" experiments of Swann *et al.* [33]. They determined the effect on tumour incidence of a variable interval between two 16 mg/kg doses of DMN compared to a single dose of either 32 or 16 mg/kg. Rats were hypersensitive to tumorigenic stimulus (i.e., developed more tumours than a single combined dose of 32 mg/kg) on days 1 and 4 after the first administration, and such sensitivity was significantly reduced by day 8. This pattern of tumour development is precisely correlated with the presence of single stranded regions in renal DNA (Fig. 2). It would appear that single stranded regions, generated by incomplete DNA repair and/or DNA replication predisposed the tissue to tumour development.

From this study the conspicuous feature of the kidney as target organ, in contrast to the non-target liver, is the occurrence of cell replication at a time when structural repair to DNA is incomplete. We suggest that this incomplete repair may be due to a specific class of carcinogen adducts, including O^6-methylguanine, which require "long patch" enzymic repair [34]. The target organ is incapable of completing this repair in terms of complete structural restoration of DNA because of enzymic deficiencies which are not necessarily confined to the appropriate endonuclease. Replication of DNA subject to incomplete excision repair is suggested by the present data as critical to the carcinogenic process. The consequences of such replication in relation to carcinogenesis have been widely discussed (see reviews [27, 32]).

The concept that cell proliferation is important in carcinogenesis is not new, being well established with demonstration of two-stage carcinogenesis in skin [35]. Farber [36], presenting a model for liver carcinogenesis, specifically related cell proliferation to the toxic and DNA damaging functions of the dietary carcinogen. Compared to these systems, induction of renal neoplasms by a single dose of DMN may exemplify a "simple" mechanism applicable only to a particular class of potent carcinogens. It is possible that such carcinogens are characterised by their ability not only to modify DNA, but also to induce specific proliferative activity in a target organ.

REFERENCES

1. R. Montesano and P. N. Magee, Comparative metabolism *in vitro* of nitrosamines in various animal species including man. In *Chemical Carcinogenesis Essays*. (Edited by R. Monresano and L. Tomatis) p. 39. IARC, Lyon (1974).
2. B. W. Stewart, Short-term carcinogen-induced structural changes in DNA *in vivo*. In *Screening Tests in Chemical Carcinogenesis*. (Edited by R. Montesano, H. Bartsch and L. Tomatis) p. 343. IARC, Lyon (1976).
3. P. H. T. Huang and B. W. Stewart, DNA damage and repair in intact animals. Single stranded regions in rat liver DNA following administration of dimethylnitrosamine. *Cancer Lett.* **1,** 263 (1976).
4. N. A. Caffin and A. G. Mackinlay, Fractionation of DNA on benzoylated DEAE-cellulose. *Analyt. Biochem.* **63,** 442 (1975).
5. G. Pirro and H. Feldmann, Characteristics of DNA fractionated on benzoylated-DEAE-cellulose. *Hoppe-Seylers Z. physiol. Chem.* **356,** 1693 (1975).
6 P. H. T. Huang and B. W. Stewart, Dimethylnitrosamine-induced structural damage to DNA results from repair of O^6-methylguanine rather than repair of 7-methylguanine. *Cancer Lett.* **5,** 161 (1978).
7. P. H. T. Huang and B. W. Stewart, Differences in patterns of structural change by rat liver DNA following administration of dimethylnitrosamine and methyl methanesulfonate. *Cancer Res.* **37,** 3796 (1977).
8. P. N. Magee and J. M. Barnes, Induction of kidney tumours in the rat with dimethylnitrosamine. *J. Path. Bact.* **84,** 19 (1962).

9. J. W. NICOLL, P. F. SWANN and A. E. PEGG, Effect of dimethylnitrosamine on persistence of methylated guanines in rat liver and kidney DNA. *Nature (Lond.)* **254,** 261 (1975).
10. R. GOTH and M. F. RAJEWSKY, Persistence of O^6-ethylguanine in rat brain DNA: correlation with nervous system specific carcinogenesis by ethylnitrosourea. *Proc. nat. Acad. Sci. (Wash.)* **71,** 639 (1974).
11. P. KLEIHUES and G. P. MARGISON, Carcinogenicity of *N*-methyl-*N*-nitrosourea: possible role of repair excision of O^6-methylguanine from DNA. *J. nat. Cancer inst.* **53,** 1839 (1974).
12. R. COX and C. C. IRVING, Selective accumulation of O^6-methylguanine in DNA of rat bladder epithelium after intravesical administration of *N*-methyl-*N*-nitrosourea. *Cancer Lett.* **3,** 265 (1977).
13. J. BUECHELER and P. KLEIHUES, Excision of O^6-methylguanine from DNA of various mouse tissues following a single injection of *N*-methyl-*N*-nitrosourea. *Chem. biol. Interact.* **16,** 325 (1977).
14. G. P. MARGISON, J. M. MARGISON and R. MONTESANO, Accumulation of O^6-methylguanine in non-target-tissue deoxyribonucleic acid during chronic administration of dimethylnitrosamine. *Biochem. J.* **165,** 463 (1977).
15. J. W. NICOLL, P. F. SWANN and A. E. PEGG, The accumulation of O^6-methylguanine in the liver and kidney DNA of rats treated with dimethylnitrosamine for a short or a long period. *Chem. biol. Interact.* **16,** 301 (1977).
16. V. M. CRADDOCK, Liver carcinomas induced in rats by a single administration of dimethylnitrosamine after partial hepatectomy. *J. nat. Cancer Inst.* **47,** 899 (1971).
17. G. THRELFALL, D. M. TAYLOR and A. T. BUCK, The effect of folic acid on growth and deoxyribonucleic acid synthesis in the rat kidney. *Lab. Invest.* **15,** 1477 (1966).
18. P. F. SWANN and A. E. M. MCLEAN, Cellular injury and carcinogenesis. The effect of protein-free high-carbohydrate diet on the metabolism of dimethylnitrosamine in the rat. *Biochem. J.* **124,** 283 (1971).
19. G. C. HARD and W. H. BUTLER, Cellular analysis of renal neoplasia: induction of renal tumors in dietary-conditioned rats by dimethylnitrosamine with a reappraisal of morphological characteristics. *Cancer Res.* **30,** 2796 (1970).
20. U. MOHR and J. HILFRICH, Effect of a single dose of *N*-diethylnitrosamine on the rat kidney. *J. nat. Cancer Inst.* **49,** 1729 (1972).
21. A. E. PEGG and J. W. NICOLL, Nitrosamine carcinogenesis: the importance of persistence in DNA of alkylated bases in the organotropism of tumour induction. In *Screening Tests in Chemical Carcinogenesis.* (Edited by R. Montesano, H. Bartsch and L. Tomatis) p. 571. IARC, Lyon (1976).
22. A. E. PEGG, Alkylation of rat liver DNA by dimethylnitrosamine: effect of dosage on O^6-methylguanine levels. *J. nat. Cancer Inst.* **50,** 689 (1977).
23. D. F. HEATH, The decomposition and toxicity of dialkylnitrosamines in rats. *Biochem. J.* **85,** 72 (1962).
24. V. M. CRADDOCK and P. N. MAGEE, Reactions of the carcinogen dimethylnitrosamine with nucleic acids *in vivo*. *Biochem. J.* **89,** 32 (1963).
25. V. M. CRADDOCK, Stability of deoxyribonucleic acid methylated in the intact animal by administration of dimethylnitrosamine. Rate of breakdown *in vivo* and *in vitro* at different dosages. *Biochem. J.* **111,** 497 (1969).
26. B. W. STEWART and P. N. MAGEE, Modification of dimethylnitrosamine-induced changes in renal metabolism and subsequent effect on carcinogenic activity of actinomycin D and cycloheximide. *Europ. J. Cancer* **9,** 37 (1973).
27. J. J. ROBERTS, The repair of DNA modified by cytotoxic, mutagenic and carcinogenic chemicals. *Advanc. Radiat. Biol.* **7,** 211 (1978).
28. D. E. SCUDERIO, E. HENDERSON, A. NORIN and B. STRAUSS, The measurement of chemically-induced DNA repair synthesis in human cells by BND-cellulose chromatography. *Mutation Res.* **29,** 473 (1975).
29. G. C. HARD, Autoradiographic analysis of proliferative activity in rat kidney epithelial and mesenchymal cell subpopulations following a carcinogenic dose of dimethylnitrosamine. *Cancer Res.* **35,** 3762 (1975).
30. G. THRELFALL, Cell proliferation in the rat kidney induced by folic acid. *Cell Tiss. Kinet.* **1,** 383 (1968).

31. B. W. Stewart and P. N. Magee, Effect of a single dose of dimethylnitrosamine on biosynthesis of nucleic acid and protein in rat liver and kidney. *Biochem. J.* **125,** 943 (1971).
32. J. E. Trosko and E. H. Y. Chu, The role of DNA repair and somatic mutation in carcinogenesis. *Advanc. Cancer Res.* **21,** 391 (1975).
33. P. F. Swann, P. N. Magee, U. Mohr, G. Reznik, V. Green and D. G. Kaufman, Possible repair of carcinogenic damage caused by dimethylnitrosamine in rat kidney. *Nature (Lond.)* **263,** 134 (1976).
34. J. D. Regan and R. B. Setlow, Two forms of repair in the DNA of human cells damaged by chemical carcinogens and mutagens. *Cancer Res.* **34,** 3318 (1974).
35. I. Berenblum, *Carcinogenesis as a Biological Problem.* North-Holland, Amsterdam (1974).
36. E. Farber, Carcinogenesis—cellular evolution as a unifying thread. *Cancer Res.* **33,** 2537 (1973).