

DNA BINDING OF 4,4'-METHYLENE-bis(2-CHLOROANILINE) (MOCA) IN EXPLANT CULTURES OF HUMAN AND DOG BLADDER*

NARAYAN SHIVAPURKAR^a, TERESA A. LEHMAN^b, HERMAN A.J. SCHUT^a and GARY D. STONER^{a,c}

^aDepartment of Pathology, Medical College of Ohio, 3000 Arlington Avenue, Toledo, OH 43699
and ^bThe Ohio State University, Comprehensive Cancer Center, Columbus, OH 43210 (U.S.A.)

(Received 10 August 1987)

(Accepted 14 August 1987)

SUMMARY

The binding to DNA of 4,4'-methylene-bis(2-chloroaniline) (MOCA) in explant cultures of human and dog bladder was compared. The DNA binding of MOCA in both human and dog bladder explants increased with the concentration of MOCA in the medium. In both species, there appeared to be a population with high DNA binding activity and another with low DNA binding activity. Furthermore, the binding of MOCA to human bladder DNA appeared to be higher than to dog bladder DNA. The results indicate the potential of MOCA to induce genetic damage in human bladder and suggest caution in the occupational exposure of humans to this chemical.

INTRODUCTION

MOCA, a commercially important curing agent in the manufacture of polyurethane [6], is an aromatic amine with structural similarity to known human bladder carcinogens [5]. It induces lung and liver tumors in mice and rats [9,15] and bladder tumors in dogs [20]. In addition, MOCA is mutagenic in the *Salmonella typhimurium* mutagenesis (Ames) assay [11], genotoxic in mouse, rat and hamster hepatocytes [12], and positive in mammalian cell transformation assays [21]. Human exposure to MOCA occurs during its manufacture and use [22]. Also, families of workers involved in the manufacture of MOCA and residents in the area of manufacturing plants are

*This research was supported by Environmental Protection Agency Cooperative Agreement No. CR-812121 and by National Cancer Institute grants CA-09498 and CA-25907. The views expressed in this article are solely those of the authors and do not necessarily reflect the position of the U.S. Environmental Protection Agency, no official endorsement should be inferred.

**To whom reprint requests should be sent.

exposed to MOCA as indicated by the presence of MOCA in their urine [22]. Evidence of its mutagenic and carcinogenic activity in experimental systems and of human exposure to MOCA suggests the importance of determining its genotoxic potential in humans and especially in the bladder.

Tissues from several human and animal organs, including the bladder, can be maintained as explants in serum-free medium [8,16,17,19]. Explant cultures of humans and animal tissues have been used extensively for comparisons of the metabolism, DNA-binding and DNA-adduct formation of a wide variety of chemical carcinogens, including certain aromatic amines [13,16,17,19]. In general, these studies have demonstrated that the metabolism of carcinogens and the profile of carcinogen-DNA adducts in human tissues is similar to that in animal model tissues in which the carcinogen has been shown to induce cancer [1,2,13,16–19]. Most differences in metabolism between humans and animals have been quantitative rather than qualitative [13,16,17,19].

In the present study, explant cultures were used to compare the ability of MOCA to bind to DNA in human and in dog bladder. The results indicate that MOCA has potential to induce levels of genetic damage in the human bladder that are similar or higher to those in the dog, a species in which MOCA has been shown to induce urothelial cancer [20].

MATERIALS AND METHODS

Chemicals

Generally tritiated labeled MOCA (s.a. = 30 Ci/mmol) was purchased from Amersham Corporation, Amersham, U.K., and was found to be 97% pure by HPLC. Unlabeled MOCA was purchased from MRU Chemical Corporation, New York, NY, and was made 97% pure by preparative reverse phase HPLC. Hydroxylapatite (HTP-DNA, HPLC grade) was obtained from Bio-Rad (Richmond, CA) and stored at 4°C until used. [³H]Thymidine (10 Ci/mmol) and [³H]leucine (10 Ci/mmol) were obtained from New England Nuclear (Boston, MA). All solvents for chromatography or DNA isolation were HPLC grade and obtained from either Sigma Chemical Co. (St. Louis, MO), Fisher Scientific (Pittsburg, PA), or Burdick-Jackson (Muskegon, MI).

Tissues

Bladder specimens were obtained from random bred dogs maintained in our facility for use in experimental surgery. Human bladder specimens were obtained from autopsies within 3–5 h after death [19]. Only grossly normal tissues were used for these investigations.

Explant culture

Specimens of bladder from the human and dog were removed by aseptic surgical techniques and placed in ice-cold L-15 medium [10] for transport to the laboratory. Explants were prepared and cultured as described previously

[19]. Attempts were made to culture approximately the same quantity of tissue in each culture dish.

Determination of MOCA toxicity in explant cultures

Eighteen hours after placing the explants in culture unlabeled MOCA was added to the medium for 4 h at a concentration of either 0.1, 1 or 10 μM (approx. 10 $\mu\text{Ci}/60$ mm dish). Control cultures were treated with 0.2% DMSO, the solvent for MOCA. Both control and MOCA-treated explants were then incubated in fresh medium containing either [^3H]thymidine (10 $\mu\text{Ci}/\text{ml}$) or [^3H]leucine (10 $\mu\text{Ci}/\text{ml}$) for 4 h. Explant DNA and protein were isolated and the radioactivity in each fraction determined as described previously [19].

DNA binding studies

An initial study was conducted to determine the binding of MOCA to the DNA of dog and human bladder explants during various periods of incubation. Eighteen hours after placing the bladder explants in culture, [^3H]MOCA was added to the medium at a concentration of 10 μM (approx. 10 $\mu\text{Ci}/60$ mm dish). The explants were then harvested after 8, 12, 18 or 24 h of incubation. Explant DNA was then isolated and the levels of binding of MOCA to DNA were determined as described below.

In subsequent studies, bladder explants from each dog or human were cultured for 18 h, and [^3H]MOCA was added to the medium at a concentration of either 0.1, 1 or 10 μM (approx. 10 $\mu\text{Ci}/\text{dish}$). After 24 h of incubation with [^3H]MOCA (viz; the initial study showed that the binding of MOCA to both dog and human bladder DNA was highest at 18–24 h), the medium was removed and the explants rinsed twice with ice-cold phosphate buffered saline (PBS). The explants were then pooled and stored at -70°C until analyses.

DNA binding assays

DNA was isolated from the explants by hydroxylapatite chromatography [19] and the purity of the DNA samples was determined from the ratio of optical densities at 260 nm and 280 nm. DNA samples having ratios between 1.75 and 1.90 were considered to be pure. The radioactivity in the DNA was quantitated by liquid scintillation counting in 3a70B complete counting cocktail (RPI, Mount Prospect, IL). The binding levels were expressed as picomoles MOCA/mg DNA.

RESULTS

Preliminary toxicity studies showed that uptake of [^3H]thymidine into DNA of dog bladder explants was not affected by MOCA at concentrations of 0.1, 1, 10 or 100 μM (data not shown). However, at 100 μM , MOCA caused a 15% inhibition in the uptake of [^3H]thymidine into the DNA of human bladder explants. Similarly, the uptake of [^3H]leucine into protein of dog

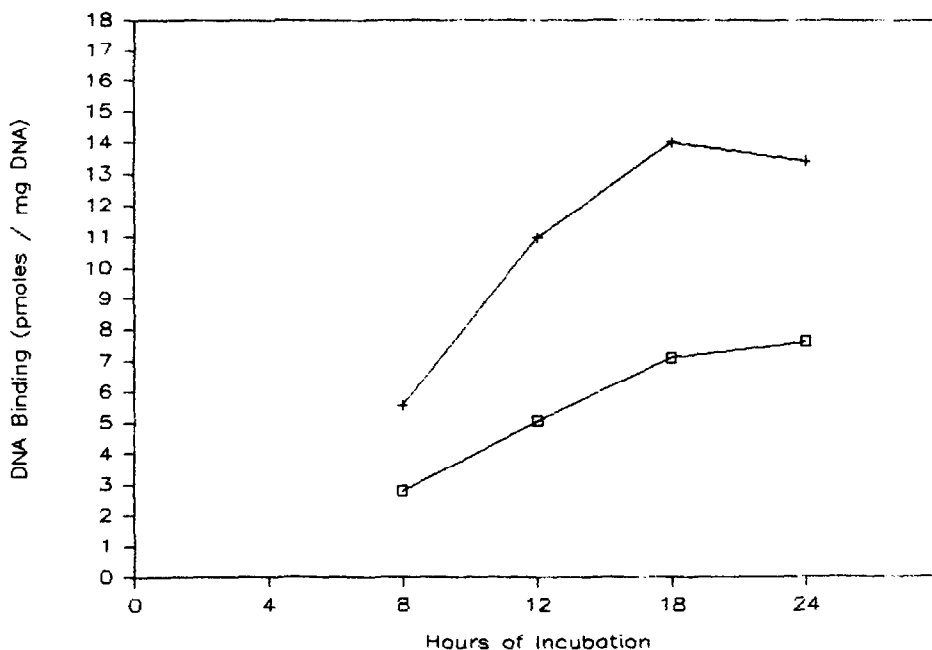


Fig. 1. Binding of [^3H]MOCA to DNA from two individual dog bladders (+, -□-). Explants were incubated with [^3H]MOCA ($10\ \mu\text{M}$ final concentration) as described in Materials and Methods. After 8, 12 and 24 h DNA was isolated from the explants by hydroxylapatite chromatography and its mass and associated radioactivity were quantitated as described in Materials and Methods.

bladder explants was not affected by any of the concentrations of MOCA tested, whereas the uptake of [^3H]leucine into protein of human bladder explants was inhibited approximately 20% by MOCA at both 10 and $100\ \mu\text{M}$. On the basis of these results, all subsequent studies with MOCA were conducted at concentrations of 0.1 , 1 and $10\ \mu\text{M}$.

Figure 1 illustrates the binding of [^3H]MOCA to the DNA of bladder explants from two dogs during 24 h of incubation. The binding of MOCA metabolites to the DNA increased with time up to 18 h and appeared to level off between 18 and 24 h. Therefore, in all subsequent studies, explants were incubated with MOCA for a period of 24 h.

Table 1 shows binding levels of [^3H]MOCA to the DNA of dog bladder explants. In both dogs as well as in humans, the amounts of MOCA bound to the DNA increased with the concentration of MOCA in the medium. However, this increase was not linear with increase in the dose from $0.1\ \mu\text{M}$ MOCA to $100\ \mu\text{M}$ MOCA. Both dogs and humans varied considerably in their susceptibility to the binding of MOCA to bladder DNA. There appeared to be a group with high binding activity and another group with low binding activity. For example,

TABLE 1

BINDING LEVELS OF MOCA TO THE DNA OF DOG AND HUMAN BLADDER EXPLANTS

Bladder case	Dog μM MOCA			Human μM MOCA		
	0.1	1.0	10	0.1	1.0	10
1	0.063 ^a	0.26 ^a	2.55 ^a	— ^b	0.36 ^a	5.03 ^a
2	0.024	0.37	1.74	—	0.37	— ^b
3	0.240	1.19	4.87	3.27	38.00	60.60
4	0.280	0.60	10.19	1.89	3.98	59.49
5	0.067	0.39	6.12	0.085	0.26	3.34
6	— ^b	0.56	— ^b	0.011	— ^b	—
7	—	—	—	0.26	—	—
8	—	—	—	—	—	5.15
9	0.72	2.32	15.04	—	—	8.56
10	0.33	0.76	5.20	—	—	11.92
11	0.13	1.18	3.40	—	—	31.95
12	—	0.36	3.60	—	—	—
13	0.12	1.88	7.49	—	—	—
14	0.035	0.80	4.03	—	—	—
15	0.61	1.00	15.96	—	—	—
16	0.21	2.95	58.08	—	—	—
17	0.10	1.43	10.33	—	—	—
18	0.27	4.37	36.62	—	—	—
Mean \pm S.D.	0.25 \pm 0.20	1.10 \pm 1.12	15.33 \pm 14.01	1.10 \pm 1.43	8.59 \pm 16.51	24.45 \pm 23.25

^aBinding is expressed as picomoles/mg DNA.^bTissue sample size was inadequate for determining binding at the concentrations indicated.

when dog bladder was incubated with MOCA at a concentration of 10 μM , the binding levels ranged from 1.74 to 58.08 pmol/mg DNA; i.e. a 33-fold variation in binding levels. In humans, at the same dose, the binding ranged from 3.34 to 60.6 pmol/mg DNA; i.e. an 18-fold variation in the binding levels.

When the binding levels of [^3H]MOCA to the DNA of dog and human bladder tissues were compared at all dose levels by analysis of variance, the binding of MOCA to the DNA of human bladder was significantly higher than that in dog bladder (F value = 7.77, $\text{PR} > F = 0.0138$). In addition, in both species, the differences in DNA binding between the three concentrations were significant (F value = 15.93, $\text{PR} < F = 0.004$). The increase in DNA binding between dogs and humans appears to be comparable when the MOCA concentration was increased from 0.1 μM to 1 μM . However, when the concentration was increased from 1 to 10 μM , the increase in binding in the human bladder (4-fold) was less than in the dog (15-fold).

DISCUSSION

The aromatic amines, like many organic carcinogens, are not direct-acting genotoxins [20]. In mammalian species, aromatic amines are metabolized and conjugated predominantly in the liver and excreted in the urine where, under mildly acidic conditions, hydrolysis of the conjugates occurs. The resulting free active metabolites may then decompose to form electrophiles that react with DNA in the bladder epithelium [7,14]. Recent studies have shown, however, that the bladder epithelium itself is capable of metabolizing carcinogens, including 2-acetylaminofluorene, to genotoxic electrophiles [2,13,16,17,19].

In the present study, we have shown that both human and dog bladder urothelium is capable of metabolizing MOCA into forms that bind to DNA. The data indicate that the binding of MOCA to human bladder DNA is higher than in dog bladder DNA. This result is of particular importance since MOCA has been shown to induce urothelial cancer in dogs [20]. The data also show a wide variation among both humans and dogs in the levels of binding of MOCA metabolites to bladder DNA. The observation is consistent with previous results in which there was a wide variation in the binding of carcinogens to human and animal bladder DNA [3,4,19]. It has been suggested that differences among individuals in their ability to metabolize carcinogens into forms that bind to DNA might be associated with carcinogenic risk [3]. If this were the case, then some individuals exposed to MOCA are more likely to develop bladder cancer than others.

Preliminary studies to detect MOCA-DNA adducts using ^{32}P -postlabeling technique, and a combination of HPLC and radiometric techniques, show formation of 6–7 adducts in the dog bladder as well as in the human bladder (unpublished results). It appears from these studies that there are at least

three adducts in common between the dog and the human. These studies need to be expanded to include more samples before any conclusions can be drawn. Nevertheless, our results clearly indicate the potential of MOCA to induce genetic damage in the human bladder and suggest caution in the occupational exposure of humans to this chemical.

REFERENCES

- 1 Autrup, H. and Stoner, G.D. (1982) Metabolism of N-nitrosamines by cultured human and rat esophagus. *Cancer Res.*, 42, 1307—1311.
- 2 Daniel, F.B., Schut, H.A.J., Sandwisch, D.W., Schenck, K.M., Hoffman, C.O., Patrick, J.R. and Stoner, G.D. (1983) Interspecies comparisons of benzo(a)pyrene metabolism and DNA-adduct formation in cultured human and animal bladder and tracheobronchial tissues. *Cancer Res.*, 43, 4723—4729.
- 3 Harris, C.C., Autrup, H., Connor, R., Barrett, L.A., McDowell, E.M. and Trump, B.F. (1976) Inter-individual variation in binding of benzo(a)pyrene to DNA in cultured human bronchi. *Science (Wash.)*, 194, 1067—1069.
- 4 Harris, C.C., Autrup H., Stoner, G.D., Trump, B.F., Hillman, E., Schafer, P.W. and Jeffrey, A.M. (1979) Metabolism of benzo(a)pyrene, N-nitrosodimethylamine, and N-nitrosopyrrolidine and identification of the major carcinogen-DNA adducts formed in cultured human esophagus. *Cancer Res.*, 39, 4401—4406.
- 5 International Agency for Research on Cancer (1972) Evaluation of carcinogenic risk of chemicals to man, *IARC Monogr.*, 4, 80—86.
- 6 International Agency for Research on Cancer (1975), 4,4'-Methylenebis(2-chloroaniline), *IARC Monogr.*, 4, 65—71.
- 7 Kadlubar, F.F., Miller, J.A. and Miller, E.C. (1977) Hepatic microsomal N-glucuronidation and nucleic acid binding of N-hydroxy arylamines in relation to urinary bladder carcinogenesis. *Cancer Res.*, 37, 805—814.
- 8 Knowles, M.A., Hicks, R.M., Berry, R.J. and Milroy, E. (1980) Organ culture of normal human bladder: Choice of starting material and culture characteristics. In: *Methods in Cell Biology*, Vol. 21B. Normal human tissue and cell culture, pp. 257—285. Editors: C.C. Harris, B.F. Trump and G.D. Stoner. Academic Press, New York.
- 9 Kommineni, C., Groth, D.M., Frockt, I.J., Voeller, R.W. and Stanovick, P. (1978) Determination of the tumorigenic potential of methylene-bis-ortho-chloroaniline. *J. Environ. Pathol. Toxicol.*, 2, 149—171.
- 10 Leibovitz, A. (1963) The growth and maintenance of tissue cell cultures in free gas exchange with atmosphere. *Am. J. Hyg.*, 78, 173.
- 11 McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975) Detection of carcinogens as mutagenic in *Salmonella*/microsome test assay of 300 chemicals. *Proc. Natl. Acad. Sci. U.S.A.*, 72, 51, 5138.
- 12 McQueen, C.A., Maslanski, C.J., Cresenzi, S.B. and Williams, G.M. (1981) The genotoxicity of 4,4'-methylenebis-2-chloroaniline in rat, mouse and hamster hepatocytes. *Toxicol. Appl. Pharmacol.*, 58, 231—235.
- 13 Moore, B.P., Hicks, R.M., Knowles, M.A. and Redgrave, S. (1982) Metabolism and binding of benzo(a)pyrene and 2-acetylaminofluorene by short-term organ cultures of human and rat bladder. *Cancer Res.*, 42, 642—648.
- 14 Poupko, J.M., Hearn, W.L. and Radomski, J.L. (1979) N-Glucuronidation of N-hydroxy aromatic amines, a mechanism for their transport and bladder-specific carcinogenicity. *Toxicol. Appl. Pharmacol.*, 50, 479—484.
- 15 Russfield, A.B., Homburger, F., Boyer, E., Van Dongen, C.G., Weisburger, E.K. and Weisburger, J.H. (1975) The carcinogenic effects of 4,4'-methylenebis-chloroaniline in mice and rats. *Toxicol. Appl. Pharmacol.*, 31, 47—54.

- 16 Schut, H.A.J., Daniel, F.B., Schenck, K.M., Loeb, T.R. and Stoner, G.D. (1984) Metabolism and DNA adduct formation of 2-acetylaminofluorene by bladder explants from human, dog, monkey, hamster and rat. *Carcinogenesis*, 5, 1287–1292.
- 17 Selkirk, J.K., Nikbakht, A. and Stoner, G.D. (1983) Comparative metabolism and macromolecular binding of benzo(a)pyrene in explant cultures of human bladder, skin, bronchus and esophagus from eight individuals. *Cancer Lett.*, 18, 11–19.
- 18 Stoner, G.D., Daniel, F.B., Schenck, K.M., Schut, H.A.J., Sandwisch, D.W. and Gohara, A.F. (1980) DNA binding and adduct formation of aflatoxin B₁ in cultured human and animal tracheobronchial and bladder tissues. *Carcinogenesis*, 3, 1345–1348.
- 19 Stoner, G.D., Daniel, F.B., Schenck, K.M., Schut, H.A.J., Goldblatt, P.J. and Sandwisch, D.W. (1982) Metabolism and DNA binding of benzo(a)pyrene in cultured human bladder and bronchus. *Carcinogenesis*, 3, 195–201.
- 20 Stula, E.E., Sherman, H., Zapp, J.A., Jr. and Clayton, J.W., Jr. (1977) Urinary bladder tumor in dogs from 4,4'-methylenebis(2-chloroaniline) (MOCA). *J. Environ. Pathol. Toxicol.*, 1, 31–50.
- 21 U.S. Dept. of Health and Human Services (1983) Public Health Service. Editor: J.E. Huff. P7.NTP Technical Bulletin No. 9.
- 22 William, D.E. (1979a) Progress report on the analysis and control of exposure to 4,4'-methylenebis(2-chloroaniline). Michigan Department of Public Health, Division of Occupational Health, Division of Environmental Epidemiology.