Europ. J. Cancer, Vol. 16, pp. 481–486
Pergamon Press Ltd. 1980. Printed in Great Britain

The Persistence in *Xenopus laevis* DNA of O^6-Methylguanine Produced by Exposure to *N*-Methyl-*N*-Nitrosourea*

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Abstract—Xenopus laevis (*the South African clawed toad*) *appears to be insensitive to the carcinogenic action of the alkylation agent* N-*methyl*-N-*nitrosourea* (*NMU*), *which induces tumours in many tissues of the rat, mouse, and other mammals. The amounts of* N^7*-methylguanine and* O^6*-methylguanine produced in* Xenopus *liver and kidney DNA by NMU injection were measured; since NMU is most effective as a carcinogen in rat tissues which cannot remove the* O^6*-methylguanine from their DNA, the persistence of these methylated purines in the* Xenopus *DNA was also studied. In* Xenopus *liver and kidney DNA the amounts of* N^7*-methylguanine and* O^6*-methylguanine found* 5 hr *after injection were comparable with the amounts found in rats after a carcinogenic dose of NMU. There was no evidence of loss of* N^7*-methylguanine or excision of* O^6*-methylguanine from either* Xenopus *liver or kidney DNA: there were no marked differences between the amounts of these purines present in liver and kidney DNA at* 5 hr *and at* 72 hr *after NMU injection.*

INTRODUCTION

N-METHYL-*N*-NITROSOUREA (NMU), a potent carcinogen in mammals, induces tumours in a variety of sites depending on dose and route of administration [1]. For example, in rats tumours were found in the gastro-intestinal tract, the skin or the kidney in 90% of the surviving animals, 3–6 months after a single oral dose (90 mg/kg body weight) of NMU [2]. By contrast, none of the *Xenopus* given NMU as single high doses (100–120 mg/kg) or multiple low doses (5 × 10 mg/kg) in our experiments during the past 5 yr have yet developed tumours in any organ [3].

Unlike the related carcinogens dimethylnitrosamine and diethylnitrosamine, the injection of which has also not resulted in tumour induction in *Xenopus* [4], NMU does not require enzymic activation, but directly alkylates DNA *in vivo*. The major product, N^7-methylguanine, is apparently irrelevant to the induction of tumours in mammals, but a minor product, O^6-methylguanine, is believed to play a critical role in both the mutagenic and carcinogenic effects of NMU [5, 6]. O^6-Methylguanine is attacked and possibly excised from DNA by an enzyme system present in the tissues of rats [7], mice [8] and Syrian hamsters [9]. There is a wide variation in the activity of this enzyme system in various organs, and there is evidence that organs with the most rapid excision of O^6-alkylguanine are least susceptible to the carcinogenic action of the nitrosamides and nitrosamines [10–12]. Since *Xenopus* appear not to develop tumours after exposure to NMU, we decided to examine the occurrence and persistence of N^7-methylguanine and O^6-methylguanine in the DNA of NMU-treated *Xenopus*.

Accepted 28 August 1979.
*This work was supported by grants from the Cancer Research Campaign.

MATERIALS AND METHODS

Animals

Adult female *Xenopus laevis* (the South African clawed toad) caught in the wild, were obtained from Xenopus Ltd., Reigate, Surrey, England, maintained in water at about 23°C and fed on chopped beef liver.

NMU

N-methyl-*N*-nitrosourea was synthesised by nitrosation of *N*-methylurea [12]. *N*-[^{14}C-methyl]-*N*-nitrosourea (1.1 mCi/mmole) was synthesised by the nitrosation of [^{14}C]methylurea which had been made from [^{14}C]methylamine by the method of Cox and Warne [13].

Elimination of [^{14}C-*methyl*]*NMU from* Xenopus

NMU is very rapidly eliminated from *Xenopus* if they are placed immediately after anaesthetisation and injection in the relatively large volumes of water in which these totally-aquatic amphibians are normally kept. To discover whether this loss could be reduced if the *Xenopus* were kept in a smaller volume of water, six adult female *Xenopus* (each weighing 75–100 g) were anaesthetised in 0.5% MS222 (tricaine methanesulphonate, Sandoz, Basel, Switzerland), given an intraperitoneal injection of [^{14}C-methyl]NMU (130 μCi, 12 mg/100 g body weight) dissolved in 0.5 ml of 0.1M phosphate buffer (pH 5.3), then placed in pairs in tanks each containing 150 ml water (pH 7.3). Water samples were taken at regular intervals, total radioactivity measured by scintillation counting, and the proportion of that radioactivity present as NMU measured after separation by thin layer chromatography (on Merck silica gel 60 F_{254} pre-coated 20 × 20 cm plates) using hexane:diethyl ether:dichloromethane:acetic acid (40:30:20:1 v/v/v/v) as solvent. The position of the NMU was detected with an u.v. lamp, the spot was scraped off, and the amount of radioactivity in it determined by scintillation counting.

Methylation of Xenopus *liver and kidney DNA by NMU*

Twenty adult female *Xenopus* (each weighing about 100 g) were anaesthetized in 0.5% MS222 and given single i.p. injections of [^{14}C-methyl]NMU (130 μCi, 12 mg/100 g) dissolved in 1 ml of 0.1 M phosphate buffer (pH 5.3). After treatment, the 20 animals were placed in two tanks, each containing 30 ml water per animal, and allowed to recover. After 6 hr, the small volume of water was increased to about 400 ml per animal. Four animals were killed at 5, 11, 24, 48 and 72 hr after injection, when their livers and kidneys were removed, immediately frozen in liquid nitrogen, then stored at −70°C until required for DNA extraction.

Extraction and analysis of DNA

DNA was prepared from the pooled kidneys or livers of each group of four *Xenopus* by phenol extraction [14] and 4–6 mg was hydrolysed in 0.1 N HCl (79°C, 30 min). Purine bases were separated on an Aminex-A6 column (6.5 × 250 mm) and eluted with 0.4M ammonium formate (pH 5). The column was maintained at 50°C. The pattern and speed of elution from Aminex-A6 is similar to that from Aminex-A7 [15], but the column of A6 can be fed at low pressure using an ordinary peristaltic pump. A typical elution pattern for alkylated *Xenopus* liver DNA hydrolysate is shown in Fig. 1. This column procedure for the analysis of alkylated DNA has been used routinely for the past three years, and gives results comparable to those obtained by chromatography on Sephadex G-10.

The amounts of guanine and adenine were estimated by measurement of the absorbance at 260 nm, assuming E_{260} to be 8000 for guanine and 13,600 for adenine. (The fractions containing guanine were made acid by the addition of HCl before the absorbance was read.) Scintillation fluid [toluene : triton X100 (3 : 2 v/v), containing 3.6 g/l diphenyloxazole] was added to each fraction (1.0 ml) and the radioactivity determined by scintillation counting. The efficiency of counting was estimated by the addition of toluene of known radioactivity (Packard Instrument Co., La Grange, Illinois, U.S.A.). The amount of each alkylated base was estimated from the radioactivity, assuming that these products would have the same specific activity as the [^{14}C-methyl]NMU.

RESULTS

The rate of loss of *N*-methyl-*N*-nitrosourea from *Xenopus* can be greatly reduced if the animals are placed in the smallest possible quantity of water after being treated. When two adult *Xenopus* were put into a tank containing only 150 ml water, approximately 50% of the injected radioactivity was still present within the body 1 hr after the dose, and 60% of the radioactivity in the water was present as NMU (Fig. 2). NMU spontaneously decomposes in water [1], so that after 4 hr, when more than 90% of the injected radioactivity had been eliminated from

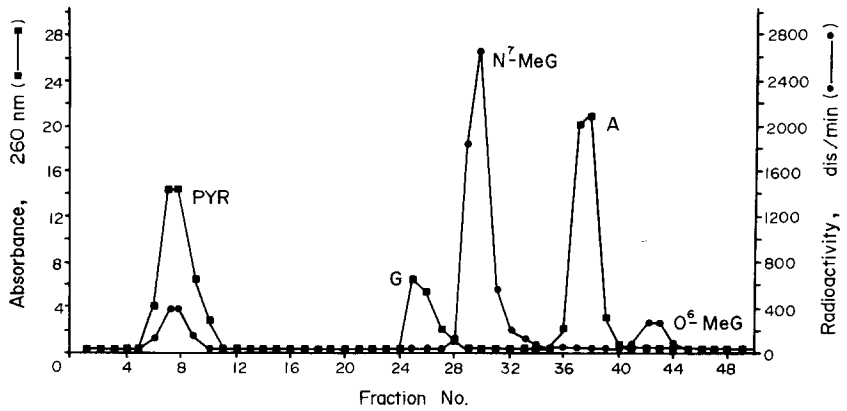

Fig. 1. *Typical elution pattern of alkylated* Xenopus *liver DNA hydrolysate on Aminex-A6* (18 ml/hr, 1.0 ml *fractions*). PYR: *pyrimidine nucleotides;* G: *guanine;* A: *adenine;* N^7*-MeG:* N^7*-methylguanine;* O^6*-MeG:* O^6*-methylguanine.* 3*-methyl adenine eluted in fractions Nos.* 128–135.

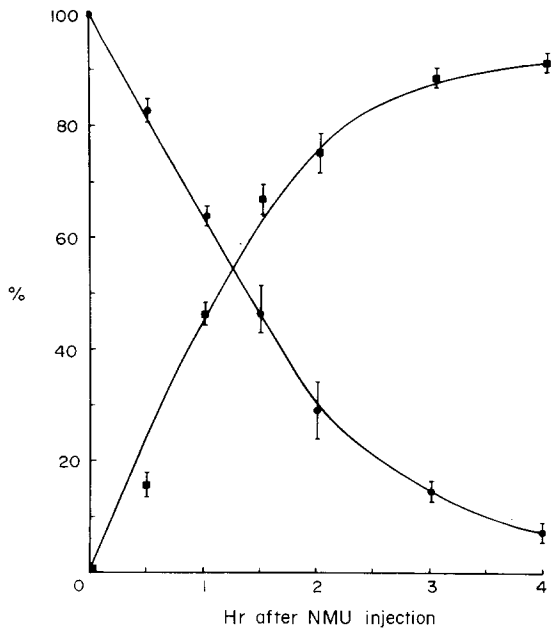

Fig. 2. *Elimination and breakdown of* N*-methyl-*N*-nitrosourea injected into adult* Xenopus. *Pairs of adult* Xenopus (*total weight* 150–200 g) *were given* [^{14}C-*methyl*]*NMU* (130 μCi, 12 mg/100 g *body weight*) *and placed in* 150 ml *water. The amount of radioactivity in the water, and the proportion of that activity present as NMU were determined as described under Materials and Methods.* ■—■, *radioactivity in water* (% *total injected*); ●—●, *radioactivity as NMU* (% *total in water*); *values given are mean*±S.E., n=3.

the body into the tank water, only 7% of the radioactivity in the water was still NMU.

The amounts of N^7-methylguanine and O^6-methylguanine in the liver and kidney DNA of *Xenopus* killed at various times after they had been given [^{14}C-methyl]NMU (12 mg/100 g) were measured. Each result in Table 1 comes from a single analysis on a DNA sample from the pooled organs of a group of four animals. The amounts of both N^7-methylguanine and O^6-methylguanine were usually higher in kidney DNA than in liver DNA. In neither tissue had the amounts of either N^7-methylguanine or O^6-methylguanine fallen greatly by the end of the 72-hr period. Table 1 also shows the values obtained for the ratio of O^6-methylguanine to N^7-methylguanine at each sample time. In both tissues the ratio increased only slightly during the 72-hr period.

DISCUSSION

The amounts of N^7-methylguanine and O^6-methylguanine found in liver and kidney DNA of *Xenopus* 5 hr after they had been given *N*-methyl-*N*-nitrosourea were similar to those found 6 hr after NMU injection in the brain, liver and kidney of the rat [10] and were above the minimum levels which appear to be required for tumour induction in some organs of mammals [8].

N^7-Methylguanine is lost from DNA by spontaneous hydrolysis [6], so the O^6-MeG/N^7-MeG ratio would have been expected to rise more markedly during the experimental period if there were no loss of O^6-methylguanine from *Xenopus* liver and kidney DNA. However, the amount of N^7-methylguanine in both tissues at 72 hr was almost as great as that present 5 hr after NMU injection. Loss of N^7-methyguanine may have been obscured by variations in the absolute amount of alkylation produced in different

Table 1. The amounts of N^7-methylguanine (N^7-MeG) and O^6-methylguanine (O^6-MeG) found in Xenopus *liver and kidney DNA at various times after a single dose of* 120 *mg/kg body weight* N-*methyl*-N-*nitrosourea*

Organ	Sample time (hr)	Methylated bases (mole % of G) N^7-MeG	O^6-MeG	O^6-MeG/N^7-MeG
Liver	5	0.155	0.014	0.090
	11	0.161	0.016	0.099
	24	0.140	0.015	0.107
	48	0.164	0.017	0.104
	72	0.146	0.017	0.118
Kidney	5	0.237	0.022	0.091
	11	0.210	0.019	0.092
	24	0.154	0.015	0.100
	48	0.204	0.022	0.109
	72	0.188	0.019	0.101

animals. These relatively large variations probably reflect the difficulty of distributing the unstable nitrosamide from the site of injection. The possibility that the amount of N^7-methylguanine (or O^6-methylguanine) was being added to by alkylation continuing well into the 72-hr period can be discounted, since 90% of the injected radioactivity would have been eliminated from the treated *Xenopus* within 4 hr, at which time only a very small proportion of the original radioactivity would have remained as NMU.

The organs of the rat which are most susceptible to the carcinogen seem to be those in which the O^6-alkylguanine persists for the greatest time. Rat brain and kidney are more susceptible to tumour induction by NMU than rat liver, and it has been suggested that this is due to the comparatively slow rate of excision of O^6-methylguanine from their DNA after exposure to the carcinogen [10]. The amounts of O^6-methylguanine remaining in rat brain and kidney at 72 hr were 67% and 50% of those present at 6 hr, but in the liver only 16% remained. From studies such as this it has been inferred that any tissue which cannot excise O^6-methylguanine from DNA would be susceptible to the carcinogenic action of the nitrosamides. If this were true, and were this the only factor involved, then tumours might have been expected in the livers and kidneys of the *Xenopus* injected with NMU in our previous experiments, since O^6-methylguanine is not excised at an appreciable rate from either of these organs. In these experiments, begun 5 yr ago, immature or adult *Xenopus* were anaesthetised in MS222 or in 5% ethyl carbamate, injected with NMU, then allowed to recover in small volumes of water for 2–3 hr. Under these conditions there would have been levels of DNA alkylation comparable with those reported in the present paper, but no tumours have yet been found in any organ of any of the animals concerned.

The rate of excision of O^6-alkylguanine is not the only important factor; there is evidence that the rate of cell proliferation is also important [16]. For example, during the increase in DNA synthesis which follows partial hepatectomy, rat liver becomes susceptible to the carcinogenic action of NMU [17]. Although relatively little cell proliferation occurs in the liver or kidneys of immature or adult *Xenopus* [18], we do not consider that this explains why NMU-treated *Xenopus* have so far failed to develop tumours. Our earliest experiments involved newly-metamorphosed toadlets which have a more rapid growth rate and therefore more extensive cell proliferation than adults, and the repeated intragastric administration of NMU to adult *Xenopus* would have been expected to result in contact between the carcinogen and proliferating cells of the gastro-intestinal tract [2]. Moreover, we examined the spleens of the *Xenopus* used in the DNA alkylation experiment and found a considerable amount of lymphoid cell death after 24–48 hr, but many mitotic figures in the white pulp after 72 hr as the lymphoid cell population began to be repopulated. There is evidence that NMU enters the cells of virtually all tissues, producing approximately the same amounts of DNA alkylation [8, 12, 19]. Thus, though we have not examined the persistence of O^6-methylguanine in organs other than liver and kidneys, the methylated

purine would have been expected to have been present during DNA synthesis in a number of tissues in the NMU-treated *Xenopus*, including the epithelial lining of the gastrointestinal tract and the lymphoid tissues, which are among the sites of tumour induction in rats and mice [2, 20, 21].

Our results with *Xenopus* do not appear to be consistent with the view that the persistence of O^6-methylguanine in a vertebrate tissue inevitably leads to the origin and development of tumours in that tissue. It is true that the maximum recorded life-span of *Xenopus* (15 yr) is longer than the 4.6 yr recorded for the rat [22], so there may be a longer latent period between the early events under discussion here and the overt appearance of tumours. We are maintaining a number of NMU-treated *Xenopus* on a long-term basis in case tumours appear late in their life-times. However, in dogs, which have a maximum recorded life-span of 20 yr [22], tumours were found only 10–15 months after the beginning of NMU treatment [23].

It is also possible that, although cell transformation and the initiation of neoplasia does occur in *Xenopus*, other control mechanisms may intervene more successfully at later stages of development in *Xenopus* than in mammals, with the result that the transformed cells are unable to develop into large malignant populations [24]. There is evidence that the incidence of spontaneous tumours may be significantly less in amphibians than in other vertebrates, and there are few (if any) convincing reports of the chemical induction of malignant tumours in amphibians [3]. In the case of the polycyclic hydrocarbons and nitrosamines this may be because their rate of elimination from the body is too fast and their rates of metabolic activation too slow for significant interaction between the ultimate carcinogen and susceptible cells to occur [4], but these explanations cannot be applied to our experiments with nitrosamides.

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