REPAIR OF O^6-METHYLGUANINE IN RAT LIVER DNA IS ENHANCED BY PRETREATMENT WITH SINGLE OR MULTIPLE DOSES OF AFLATOXIN B_1

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Summary.—Pretreatment of rats by the repeated administration of certain alkylating carcinogens has been shown to stimulate the removal of O^6-alkylguanine from hepatic DNA. Prolonged feeding with the aromatic amide 2-acetylaminofluorene has a similar effect. In this report, aflatoxin B_1, an agent from another chemically distinct class of carcinogen, is shown to be capable of stimulating the repair of O^6-methylguanine in hepatic DNA. The sensitivity of this system is shown by the fact that this repair response can be fully stimulated as early as 1 day after treatment with a single dose.

IT IS NOW RECOGNIZED that in some regions of the world where food-spoilage by fungi is a problem, aflatoxins formed by the mould *Aspergillus flavus* can lead to prolonged human exposure and may be hepatocarcinogenic (Wogan, 1976). The question arises whether their presence might alter the response to other factors, and in this connection it is important to evaluate the possible role of additional carcinogenic components in the environment. The following studies on the repair of O^6-methylguanine reveal an interesting effect that could be relevant to the action of another important group of environmental carcinogens, the nitroso compounds. In view of the current debate on the potential importance of the roles of different environmental carcinogens (*i.e.* those associated with lifestyle or occupation, Anon., 1981), observations of the kind made in this communication may be of interest.

Reports have already indicated that the chronic treatment of rats with dimethylphenyltriazene (DMPT, Cooper *et al.*, 1978), dimethylnitrosamine (DMN, Mon-

tesano *et al.*, 1979) or diethylnitrosamine (DEN, Margison *et al.*, 1979) can lead to an enhanced repair of O^6-alkylguanine in hepatic DNA, and that prolonged treatment with 2-acetylaminofluorene (AAF) will produce a similar effect (Buckley *et al.*, 1979). As the two classes of carcinogen used in these pretreatments are very different in chemical structure, it was important to determine whether agents from other chemically distinct classes of carcinogen are capable of inducing a similar response. These results show that chemically unrelated compounds (*i.e.* nitrosamines, an aromatic amide and a fungal product, aflatoxin), which are known to be hepatoxic but activated by different mechanisms, are all capable of enhancing the repair of O^6-methylguanine.

MATERIALS AND METHODS

Materials.—Di[^{14}C]-methylamine hydrochloride (54 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks, and used to prepare di-[^{14}C]methylnitrosamine (^{14}C-DMN) by the method of Dutton & Heath (1956); unlabelled nitrosamine was

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supplied by Eastman Kodak Ltd, Kirkby, Lancs. Aflatoxin B₁ (AFB_1) was purchased from Sigma Chemical Co., London, and Sephadex G10 was from Pharmacia (G.B.) Ltd. Wistar rats of the strain maintained in these laboratories were used.

Methods. — Male rats (220–240 g) were given i.p. injections of AFB_1, suspended in a solution of gum acacia (1·75% in saline); control animals received vehicle alone. At various times later they were given i.p. injections of ¹⁴C-DMN (2 mg/kg; sp. act. 3–8 mCi/mmol) prepared in saline. After 5 h the animals were killed, and the liver removed. In each case a small sample of tissue was fixed in formalin, embedded in paraffin, sectioned, and stained with haematoxylin and eosin. The remainder was frozen on to dry ice and stored at −30°C for isolation of DNA.

DNA was extracted by a modified phenol procedure (Kirby & Cook, 1967) and hydrolysed in 0·1M HCl (70°C, 30 min). Authentic marker compounds 7-methylguanine, 3-methyladenine and O^6-methylguanine were added and the pH of the hydrolysate adjusted to 2·95. Purine bases were separated by chromatography (Lawley & Shah, 1972; Margison *et al.*, 1976) on columns of 1 × 100cm Sephadex G-10 eluted with 0·05M ammonium formate–0·02% (w/v) sodium azide (pH 6·75) at a flow rate of 15–20 ml/h. The absorption at 260 nm of each fraction (4·5 ml) was determined, from which the amounts of adenine and guanine were calculated. The samples were dried and counted for radioactivity after addition of water (0·5 ml) and Triton-toluene phosphor (5 ml). These measurements were used to estimate the amounts of methylated purines, on the assumption that the specific activity of the ¹⁴C-labelled methyl group had remained unchanged.

RESULTS

Effect of single doses

In the first of these experiments (Table I) rats were treated with AFB_1 at doses which span the half LD_{50} range for the rat (IARC, 1976). Twenty-four hours later these animals were challenged with a low dose (2 mg/kg) of ¹⁴C-DMN, and after 5 h the amounts of methylpurines formed in hepatic DNA were measured. There was no obvious effect of the pretreatment upon the rate of metabolism of DMN, as the amounts of N7-methylguanine present were similar for all pretreatment doses, but changes were detected in the amounts of O^6-methylguanine and 3-methyladenine in DNA. At a pretreatment dose of 2 mg/kg, there was a clear enhancement of the repair of O^6-methylguanine, which is indicated by ratios of O^6-methylguanine to 7-methylguanine, that are less than the

TABLE I.—*Amounts of methylpurines in liver DNA of Wistar rats treated with varying doses of AFB_1, 24 h before administration of ¹⁴C-DMN (2 mg/kg). Each analyses was made on DNA from the liver of an individual animal killed 5 h after injection of the nitrosamine. The figures in parentheses are the amounts of O^6-methylguanine (O^6-meG) or of 3-methyladenine (3-meA) relative to 7-methylguanine (7-meG)*

AFB_1 (mg/kg)	O^6-meG	3-meA	7-meG	d/min/ μmol A*	d/min/ μmol G*
		(μmol/mol parent base)			
0	48·4 (0·081)	18·3 (0·030)	600·6	2·8	4·6
	45·0 (0·072)	19·1 (0·031)	622·9	11·2	13·3
0·5	43·3 (0·061)	23·5 (0·033)	706·1	13·5	15·9
	60·9 (0·077)	26·1 (0·033)	792·8	15·0	14·5
2·0	28·1 (0·037)	22·1 (0·029)	760·2	40·7	50·7
	24·0 (0·031)	22·8 (0·030)	771·3	77·9	105·9
6·0	67·2 (0·089)	37·9 (0·050)	751·3	36·6	65·6
	59·1 (0·106)	29·0 (0·052)	556·2	12·1	16·9
12·0	75·3 (0·115)	40·5 (0·062)	651·7	12·1	12·1
	67·2 (0·095)	33·6 (0·047)	708·7	27·1	31·5

* These values are corrected for differences in the specific radioactivity of the ¹⁴C-DMN by dividing the actual value by the specific activity of the batch of isotope used.

TABLE II.—*Changes in amounts of methyl purines in liver DNA of Wistar rats pretreated with AFB_1 (2 mg/kg) and then given ^{14}C-DMN (2 mg/kg) at different times later. (a) At the appropriate interval after pretreatment with AFB_1 or vehicle, animals were given an i.p. injection of labelled nitrosamine. 5 h later they were killed, liver DNA was isolated and analysed as described in Methods. Each group comprised 2 animals except in the case of 1-day controls (3 rats) and 28-day pretreated (1 rat). Values shown are the mean of separate analyses on liver DNA of individual animals. DMN injected on basis of body weight+ when pretreated with AFB_1; ++when injected with ^{14}C-DMN. (b) Details as above except that in each case a single dose of unlabelled DMN (2 mg/kg) was administered 3 days after the AFB_1 treatment*

Condition		O^6-meG N7-meG (μmol/mol guanine)		O^6/N7	d/min/ μmol A*	d/min/ μmol G*
(a) Single dose of DMN						
Control	6 h+	56·2	735·1	0·076	5·6	5·3
	12 h+	52·3	559·0	0·093	13·9	12·4
	1 day+	47·1	616·5	0·076	7·5	9·2
	10 day++	70·1	673·3	0·104	13·6	8·8
	21 day++	71·7	659·6	0·109	14·3	12·4
	28 day++	60·4	646·9	0·093	8·4	8·3
	35 day++	65·2	736·9	0·089	14·1	12·8
Pretreated	6 h+	77·9	770·0	0·101	4·9	8·9
	12 h+	71·4	702·5	0·102	3·3	2·4
	1 day+	26·1	765·8	0·034	59·3	78·3
	2 day+	27·9	952·5	0·031	83·1	82·0
	5 day+	34·5	885·1	0·039	171·8	159·6
	10 day+	56·0	930·5	0·060	195·1	264·8
	10 day++	39·7	593·7	0·067	43·8	34·7
	15 day++	33·5	496·8	0·067	19·4	24·0
	21 day++	25·6	523·7	0·049	20·5	19·4
	28 day++	46·7	610·0	0·077	44·8	59·2
	35 day++	45·3	724·0	0·063	24·4	12·6
(b) Two doses of DMN						
Pretreated	Day 10++	20·2	504·6	0·040	132·1	121·2
	Day 18++	39·0	507·8	0·078	24·9	24·2
	Day 21++	36·8	557·7	0·066	69·5	42·3

* Values corrected as in Table I.

expected value (0·11) for this alkylating agent (O'Connor *et al.*, 1979). Below this (at 0·5 mg/kg) there was no effect, and above 2 mg/kg the toxic effects of AFB_1 may have been responsible for inhibiting the repair process. These higher doses (6 and 12 mg/kg) also inhibited the repair of 3-methyladenine, as determined from a comparison of the ratios of 3-methyl-adenine to 7-methylguanine, but at the lower doses, and in the experiments reported later, there was no change in this repair function: the methylpurine ratio remained constant at 0·03 ± 0·0015. The toxicity of the mycotoxin was also clear from the histological appearance of the liver, slight degeneration could be detected even at the lower doses, and,

above 2 mg/kg, both degeneration and some necroses were present. In keeping with this, there was some evidence of a regenerative response, as judged by the incorporation into DNA of labelled 1-C fragments from the breakdown of ^{14}C-DMN. This was increased by pretreatment with AFB_1 at doses of 2 mg/kg and above (Table I).

Duration of the repair response

Although environmental exposures to aflatoxins are often protracted, it was of interest to see how long the enhanced capacity for the repair of O^6-methyl-guanine could persist after a single treatment. In these experiments rats were pre-treated with AFB_1 (2 mg/kg) and chal-

TABLE III.—*Changes in amount of methyl purines in liver DNA of Wistar rats pretreated with* AFB_1 *(0·5 mg/kg) and then given* ^{14}C*-DMN (2 mg/kg) at different times later. The DMN dose was based on the body weight on the day of the nitrosamine injection. Each group comprised 2 rats and the values shown are the mean of analyses for the individual animals*

Conditions		O^6-meG (μmol/mol guanine)	N7-meG	O^6/N7	d/min/ μmol A*	d/min/ μmol G*
Control	1 day	46·7	611·7	0·076	7·0	8·9
	10 days	70·5	673·3	0·104	13·6	8·8
Pretreated	1 day	52·1	749·4	0·070	14·3	15·2
	2 days	26·3	555·7	0·047	9·4	13·9
	4 days	31·6	518·2	0·061	28·6	34·0
	10 days	55·6	615·9	0·090	7·7	3·2

* Values corrected as in Table I.

lenged with DMN at different times from 6 h to 35 days later (Table II). The repair response elicited by this single dose of mycotoxin occurred between 12 and 24 h after pretreatment, and persisted at a similar level for at least 5 days. After this the response was more variable, but the ratio of O^6-methylguanine to 7-methylguanine was consistently below that for the control rats even 35 days later. The control animals for the experiment, however, had a ratio of methylguanines that was rather higher than in other studies involving treatment with DMN at 2 mg/kg. This effect could be due to the injection of gum acacia. However, irrespective of these changes in control values it is evident that the enhanced response can persist for several weeks after a single dose of AFB_1, though there may be more individual variation later.

The timing of histological changes in the livers of the AFB_1-pretreated animals, in comparison with the normal histology seen in the gum acacia controls, is as follows. From 12 h until Day 15 some degeneration was present, but by Day 21 and later this effect decreased. Necroses were obvious from 2 until 10 days, when they too began to decline. Proliferation of liver cells was not noticeable until Day 5, but this then persisted to a variable degree throughout the period of observation, and bile-duct proliferation followed a similar pattern. As seen in Table I, changes in the extent of incorporation of labelled 1-C fragments into DNA purines in this experiment were also consistent with some degree of proliferative activity during this period.

The enhanced repair response can also be induced by a lower dose of AFB_1 (Table III), but the observed effect is smaller, takes longer to appear and is of shorter duration. At this lower dose there was little sign of a proliferative response, as judged by the extent of incorporation from the labelled 1-C pool. Although the livers of the pretreated animals showed signs of slight degeneration up to the 4th day after pretreatment, necroses were very rare, there was no detectable proliferation of liver cells and only one animal showed traces of bile-duct proliferation, at Day 10 when the repair-response had subsided.

Effects of multiple doses

A cumulative effect of low doses of AFB_1 was also noted. Four consecutive daily doses of 0·5 mg/kg elicited a repair response (ratio of methylguanines 0·041) similar to that produced by a single dose of 2 mg/kg (Tables I and II) when the animals were challenged with labelled DMN (2 mg/kg) 24 h after the last dose of AFB_1. On the other hand, 8 consecutive daily doses of AFB_1 (0·25 mg/kg each) produced only an equivocal response when the animals were challenged on the 9th day.

DISCUSSION

So far reports indicate that once the repair of O^6-alkylguanine has been en-

hanced, the response can persist for pro-
longed periods as the treatment is con-
tinued in the cases of DMN (Montesano
et al., 1979, 1980), DEN (Margison et al.,
1979) or AAF (Buckley et al., 1979;
Charlesworth et al., 1981) and we now show
that it can also persist for a week or more
after single treatments with AFB$_1$. In
livers in which this response is induced the
repair capacity appears to be present in
amounts sufficient to remove O^6-methyl-
guanine from DNA more efficiently than in
the controls. However, in all the earlier
reports only single challenging doses of
labelled nitrosamine were given, so there
was no indication whether the supply of
induced enzymes might be easily exhaus-
ted. In the present series of experiments
using a single dose of AFB$_1$, administra-
tion of a low dose of unlabelled DMN
(2 mg/kg) shortly after the response had
been initiated by the mycotoxin did not
alter the response when the animals were
challenged later with labelled nitrosamine
(Table II).

It is now clear that the hepatic repair
system for O^6-methylguanine can be
induced in rats by a variety of agents, i.e.
DMPT (Cooper et al., 1978), DMN (Monte-
sano et al., 1979, 1980), DEN (Margison
et al., 1979), AAF (Buckley et al., 1979)
and AFB$_1$. These agents are hepatotoxic
to varying degrees and will react covalently
with DNA after metabolism, and so
would be expected to induce some degree
of compensating regeneration. On the
basis of the histological assessment this is
indeed the case in the experiments repor-
ted here, in which proliferation of liver
cells and of bile ducts could be seen after
several days. The evidence provided by the
incorporation of ^{14}C-labelled 1-C frag-
ments into DNA purines (Tables I, II
and III) supports this conclusion, and there
is a tendency for the higher levels of 1-C
incorporation to correspond with the
lower ratios of O^6-methylguanine to 7-
methylguanine. However, this correlation
is by no means precise, and it is not pos-
sible from the present data to decide on
the role of toxicity, DNA damage or any

other factor in inducing this response. In
practice, however, this enhanced capacity
for DNA repair may afford cell protection
in an organ which has to play a major part
in the detoxification process. In this con-
text it is of particular interest to note that
an "adaptive" response may occur not
only after prolonged exposure to an agent
but as early as one day after a single treat-
ment. In more general terms, this observa-
tion has wide implications not only for
exposures of an environmental nature,
but also for those occurring during therapy.

REFERENCES

ANON. (1981) Two views of the causes of cancer.
Nature, 289, 431.
BUCKLEY, J. D., O'CONNOR, P. J. & CRAIG, A. W.
(1979) Pretreatment with acetylaminofluorene
enhances the repair of O^6-methylguanine in DNA.
Nature, 281, 403.
CHARLESWORTH, J. D., O'CONNOR, P. J. & CRAIG,
A. W. (1981) Effect of pretreatment by feeding
acetylaminofluorene on methylated purines
formed in rat liver DNA after administration of
dimethylnitrosamine. Carcinogenesis. 2, 329.
COOPER, H. K., HAUENSTEIN, E., KOLAR, G. F. &
KLEIHUES, P. (1978) DNA alkylation and neuro-
oncogenesis by 3,3-dimethyl-1-phenyltriazene.
Acta Neuropathol. (Berl.), 43, 105.
DUTTON, A. H. & HEATH, D. F. (1956) The prepara-
tion of ^{14}C-dimethylamine and ^{14}C-dimethyl-
nitrosamine. J. Chem. Soc., 7, 1892.
I.A.R.C. (1976) Aflatoxins. In Evaluation of the
Carcinogenic Risk of Chemicals to Man. Vol. 10.
Lyon: WHO. p. 51.
KIRBY, K. S. & COOK, E. A. (1967) Isolation of
deoxyribonucleic acid from mammalian tissues.
Biochem. J., 104, 254.
LAWLEY, P. D. & SHAH, S. A. (1972) Methylation of
RNA by the carcinogens dimethylsulphate,
N-methyl-N-nitrosourea, N-methyl-N'-nitro-N-
nitrosoguanidine. Biochem. J., 128, 117.
MARGISON, G. P., CURTIN, N. K., SNELL, K. &
CRAIG, A. W. (1979) Effect of chronic N,N-
diethylnitrosamine treatment on the excision of
O^6-ethylguanine from rat liver DNA. Br. J.
Cancer, 40, 809.
MARGISON, G. P., MARGISON, J. M. & MONTESANO, R.
(1976) Methylated purines in the DNA of various
Syrian golden hamster tissues after administration
of hepatocarcinogenic dose of dimethylnitro-
samine. Biochem. J., 157, 627.
MONTESANO, R., BRÉSIL, H. & MARGISON, G. P.
(1979) Increased excision of O^6-methylguanine
from rat liver DNA after chronic administration
of dimethylnitrosamine. Cancer Res., 39, 1798.
MONTESANO, R., BRÉSIL, H., PLANCHE-MARTEL, G.,
MARGISON, G. P. & PEGG, A. E. (1980) The effects

of chronic treatment of rats with dimethyl-nitrosamine on the removal of O^6-methylguanine from DNA. *Cancer Res.*, **40**, 452.

O'CONNOR, P. J., SAFFHILL, R. & MARGISON, G. P. (1979) *N*-Nitroso compounds: Biochemical mechanisms of action. In *Environmental Carcinogenesis*. Ed. Emmelot & Kriek. Amsterdam: Elsevier/North Holland Biomedical Press. p. 73.

WOGAN, G. N. (1976) Aflatoxins and their relationship to hepatocellular carcinoma. In *Hepatocellular Carcinoma*. Ed. Okuda & Peters. London: Wiley & Sons. p. 25.

Note added in proof

Cell-free extracts from the livers of rats treated with AAF or AFB$_1$ show an increased capacity to remove O^6-methylguanine from DNA in *in vitro* assays when compared to extracts from the livers of normal animals. Cooper *et al.* (1981), Maru *et al.* (1981), *Br. J. Cancer* (In press).