INTERACTION OF A CARCINOGEN, 4-HYDROXYAMINOQUINOLINE-1-OXIDE WITH NUCLEIC ACIDS

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Received October 2, 1967

The potent carcinogenic action of 4-nitroquinoline-1-oxide (4NQO) was first demonstrated by Nakahara et al. (1957), and subsequent studies of the metabolism of this compound have revealed that it is reduced to a noncarcinogenic compound, 4-aminoquinoline-1-oxide (4AQO) through the highly carcinogenic intermediate (Shirasu and Ohta, 1963; Endo and Kume, 1963), 4-hydroxyaminoquinoline-1-oxide (4HAQO) (Sugimura et al., 1965; 1966). It has been assumed that 4HAQO is the active form of the carcinogen. These carcinogenic compounds also induce mutation in microorganisms (Mashima and Ikeda, 1958; Okabayashi et al., 1965).

Studies of the inactivation of both T_4 phage (Ishizawa and Endo, 1967) and transforming ability of DNA from Bacillus subtilis (Ono et al., 1966) by 4HAQO have led to the suggestion that the carcinogenicity and mutagenicity of these compounds are due to the interaction of 4HAQO with DNA. Nagata et al. (1966), using flow dichroism, demonstrated a direct interaction of 4NQO and 4HAQO with calf thymus DNA and Malkin and Zahalsky (1966) reported evidence of a similar interaction using thin-layer chromatography.

In this communication we report the formation of specific complexes between carcinogen and DNA or RNA formed by exposure of ascites cells to 4HAQO. The DNA complexes show decreased

template ability with a purified DNA-dependent RNA polymerase system.

MATERIALS AND METHODS

4HAQO, 4AQO and 4-hydroxyquinoline-1-oxide (4HQO) were kindly provided by Professor M. Hamana, Faculty of Pharmaceutical Sciences, Kyushu University. 4NQO was obtained from Daiichi Pure Chemicals Co.. Guanosine triphosphate-8-^{14}C was purchased from Schwartz BioResearch. Albino rats bearing ascites hepatoma tumors (AH 130 line) were used for the experiments on the sixth day after the intraperitoneal transplantation.

DNA-dependent RNA polymerase (EC 2.7.7.6) was purified from Escherichia coli strain B (H) using DEAE-cellulose chromatography, ammonium sulfate fractionation, hydroxylapatite chromatography and DEAE-Sephadex chromatography, successively (Tada et al., 1967). Nucleic acids, RNase, DNase and polynucleotide phosphorylase were either absent or present in negligible amounts and the specific activity of the final preparation was approximately 6000 units/mg protein (according to the method of Chamberlin and Berg, 1962).

RESULTS AND DISCUSSION

As shown in Fig. 1, DNA isolated from 4HAQO-exposed cells was fluorescent, in contrast to DNA isolated by the same procedure from untreated cells which showed no characteristic fluorescence under the same conditions. While the fluorescence resembled that of 4HAQO, shifts were observed in both excitation and fluorescence maxima. RNA isolated from 4HAQO-exposed cells showed similar fluorescence characteristics (Table III).

The amount of fluorescent compound bound depended on the dose of administered carcinogen and was approximately 0.5 - 1.5 molecules per 1000 nucleotides of DNA, assuming that the bound

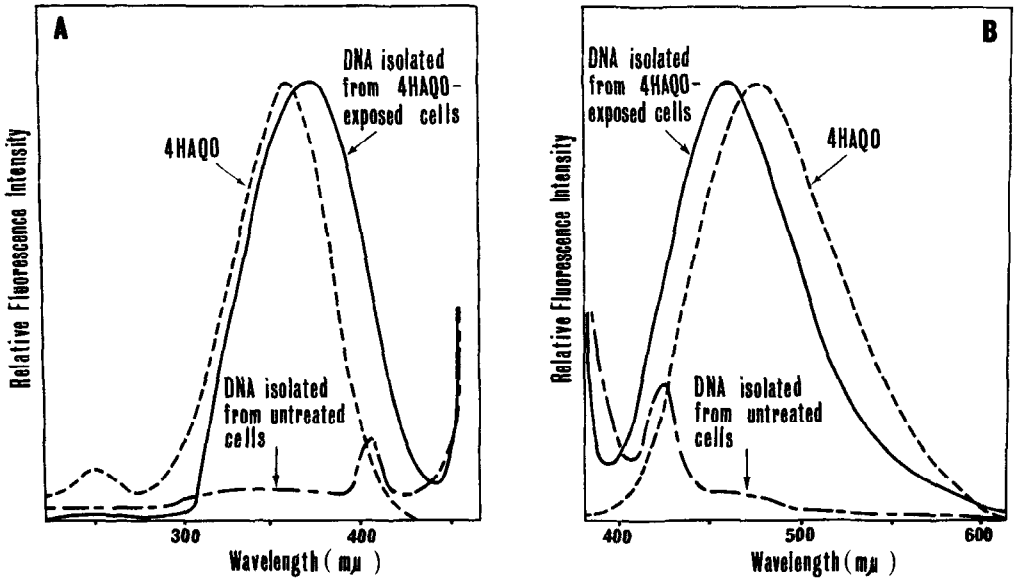

Fig. 1. Excitation (A) and fluorescence (B) spectra of 4HAQO compared with those of DNA isolated from 4HAQO-exposed cells and from untreated cells.

4HAQO was administered intraperitoneally by injection of 0.5 ml of 20 mM 4HAQO in 50% propyleneglycol to give a final concentration of 0.5 mM 4HAQO in the ascites fluid. Ascites cells were collected 60 minutes after administration and were washed 3 times with 10 volumes of Hanks' solution (Hanks and Wallace, 1949). DNA was extracted from the cells by the method of Kay et al. (1952) followed by RNase T_2 treatment and phenol extraction. DNA was washed repeatedly with ethanol and acetone to remove free 4HAQO and its derivatives. As a control, DNA was obtained from untreated cells in the same manner. The DNA preparations contained less than 1% protein. Fluorescence was measured in 1.5 mM sodium citrate (pH 7.3) containing 15 mM NaCl by a Hitachi Spectrofluorometer MPF-2. The figure shows uncorrected instrument readings. Concentrations of samples were 3 μM for 4HAQO and 1 mg/ml for DNA.

Table I

Binding of the carcinogen to DNA and its effect on the template ability of DNA in the DNA-dependent RNA polymerase system

Source of DNA	Relative fluorescence intensity at 460 mμ	GMP-^{14}C incorporation (cpm/mg DNA/hr)	Relative rate of incorporation (%)
DNA isolated from untreated cells	7	21630	100
DNA isolated from 4HAQO-exposed cells*	100	3330	15
	70	8680	40
	38	10690	49
	30	13710	63
DNA treated with 4HAQO *in vitro***	66	14180	65

*Various amounts of 4HAQO were administered intraperitoneally to give a final concentration of 0.05 - 0.5 mM in the ascites fluid. DNA was extracted from 4HAQO-exposed ascites cells as described in the legend to Fig. 1.
**DNA isolated from untreated cells was incubated with 4HAQO *in vitro* (see text).

The reaction mixture (0.25 ml) contained, in μ moles; Tris HCl (pH 7.6), 30; $Mg(Ac)_2$, 1.5; $MnCl_2$, 0.5; β-mercaptoethylamine, 1.5; 8-^{14}C-GTP, 0.1 (20 mμc); ATP, CTP and UTP, 0.1 each; 10 μg of DNA; 100 units of polymerase. After incubation for 60 minutes at 37°, radioactivity incorporated in the fraction insoluble in cold 5% TCA was measured in a windowless gas flow counter. Condition of fluorometry was as described in the legend to Fig. 1.

compound had the same fluorescence intensity as 4HAQO. There was a reciprocal relationship between the extent of binding and the template ability for DNA-dependent RNA polymerase (Table I). On the other hand, 4HAQO and 4AQO did not affect the enzyme activity in the assay system at concentrations 10 times higher than the amount present in the bound form. Therefore the loss of template activity is due to a modification of DNA than to contamination of the DNA preparation by free 4HAQO or 4AQO.

When the DNA preparations were heated to 100^{o}, 95 - 100 % of the fluorescent compound was released, and the template activity of the DNA increased to that of heat denatured control DNA (Table II).

Nucleic acids isolated from 4HAQO-exposed cells were heated and the released fluorescent compound was recovered from the supernatant solutions after ethanol precipitation. The fluorescence properties were identical with those of the bound form. As summarized in Table III, fluorometric studies showed that the fluorescent compound, both in the bound and released forms, was not identical with 4HAQO, 4AQO, 4HQO or 4NQO nor with the oxidative condensation product of 4HAQO which is formed at pH 7.3, most probably 4,4'-azoxyquinoline-1,1'-dioxide (Hamana, 1967; Ishizawa and Endo, 1967).

To compare our results with *in vitro* interaction studies reported by other workers, DNA (0.1 mg/ml) isolated from untreated cells was incubated with 0.3 mM 4HAQO in 10 mM Tris HCl (pH 7.6) at 37^{o} for 10 minutes. After repeated washing with ethanol and acetone, the resulting DNA was found to be fluorescent and the template activity for the RNA polymerase was decreased (Table II); however, the fluorescence characteristics of this treated DNA were identical with those of 4HAQO and the inhibi-

Table II

Release of fluorescent compound from DNA and recovery of reduced template ability of DNA by heating

Source of DNA		Relative fluorescence intensity at 460 mμ*	GMP-^{14}C incorporation (cpm/mg DNA/hr)	Relative rate of incorporation (%)
DNA isolated from 4HAQO-exposed cells	native	70	7780	34
	heated**	9	14740	64
DNA isolated from untreated cells	native	7	23120	100
	heated**	7	14300	62

*Relative intensity compared with that of Table I.
**DNA (0.1 mg/ml) in 1.5 mM sodium citrate (pH 7.3), containing 15 mM NaCl, was heated in boiling water for 10 minutes and cooled immediately in ice.

Assay was as described in the legend to Table I.

Table III

Fluorescence characteristics of nucleic acids isolated from 4HAQO-exposed cells, 4HAQO and its derivatives

	Excitation maxima (mμ)	Fluorescence maxima (mμ)
DNA isolated from 4HAQO-exposed cells	375	460
RNA isolated from 4HAQO-exposed cells*	375	460
Released compound**	375	460
4HAQO	360	478
4AQO	365	478
4HQO	360	480
4NQO***	360	480
Condensation product of 4HAQO****	420, 440, 470	550

*RNA was extracted from cytoplasmic fractions by SDS-phenol at room temperature (Tada and Tada, 1967).
**compound released by heating (see text).
***4NQO becomes fluorescent by radiation of 360 mμ light.
****most probably 4,4'-azoxyquinoline-1,1'-dioxide (see text).

Fluorometry was carried out as described in the legend to Fig. 1.

tion of template activity was much less than DNA exposed *in vivo*. Consequently, the DNA-carcinogen complexes formed *in vitro* must differ from those formed *in vivo*.

DNA isolated from 4HAQO-exposed cells was hydrolyzed to 3'-mononucleotides by successive incubation at 37° with micrococcal nuclease (Worthington, 100 units/mg DNA for 2 hours) and spleen phosphodiesterase (Worthington, 1.5 units/mg DNA for 2 hours) and the hydrolysate was chromatographed on a Dowex-1 column (Tada *et al.*, 1964; 1967). Approximately 50 % of the fluorescent material released by incubation passed through the column; the remaining part was eluted together with nucleotides. The RNase T_2-hydrolysate (6.5 units/mg RNA for 20 hours, Hiramaru *et al.*, 1966) of RNA isolated from 4HAQO-exposed cells showed a similar chromatographic pattern. It appears that the fluorescent compound still binds to nucleotides after enzymic digestion which suggests that the fluorescent compound is associated with nucleic acids not by intercalation but by covalent bonding.

Our results show that 4HAQO can be incorporated into ascites cells and interacts with DNA to inhibit RNA synthesis. This agrees with the previous observations on diminishing precursor uptake into nucleic acids *in vivo* by 4NQO and 4HAQO (Fukuoka and Naora, 1957; Takahashi *et al.*, 1964).

At present there is lack of general agreement as to the site of action of this carcinogen within the cell. However other carcinogenic agents, such as alkylating agents, nitroso compounds, lactones, azo-dyes and hydrocarbons, have been found to react with nucleic acids (for reference, see Brookes, 1966). Since the concentration of the carcinogen employed in our investigation is comparable to the dose at which a malignant transformation in cultured hamster cells was observed (Sato and Kuroki, 1966), it

is likely that interaction of 4HAQO with nucleic acids is related to its carcinogenicity and mutagenicity.

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