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INVESTIGATIONS ON THE RELATIONSHIP BETWEEN DNA ETHENOBASE ADDUCT LEVELS IN SEVERAL ORGANS OF VINYL CHLORIDE-EXPOSED RATS AND CANCER SUSCEPTIBILITY*

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Abstract—The levels of 1,N^6-ethenodeoxyadenosine (εdAdo) and 3,N^4-ethenodeoxycytidine (εdCyd) were measured in DNA of several target organs of vinyl chloride (VC)-exposed rats. Seven-day-old (group I) and 13-week-old (group II) BD VI rats were exposed during 2 weeks to 500 ppm VC in air (7 hr per day and 7 days per week). εdAdo and εdCyd were measured by a combination of prepurification of DNA hydrolysates by HPLC and competitive radioimmunoassay using specific murine monoclonal antibodies. Both ethenodeoxynucleosides were detected in liver, lungs and brain (levels ranging from 0.6×10^{-7} to 1.3×10^{-7} for εdAdo/2′-deoxyadenosine and from 1.95×10^{-7} to 4.92×10^{-7} for εdCyd/2′-deoxycytidine) but not in kidneys of group I rats. In group II rats, only liver DNA was analysed and the levels of each adduct were six times lower than in young (group II) rats. These findings are in good agreement with the organotropism and the age-related sensitivity of VC-induced carcinogenesis in rodents.

Occupational exposure to vinyl chloride (VC), a widely used industrial chemical, has been associated with the occurrence of angiosarcoma of the liver [1]. VC itself is inactive and its biological effects are dependent upon its metabolic conversion by the microsomal P-450-dependent monooxygenase system into chloroethylene oxide, which can rearrange non-enzymatically to chloroacetaldehyde [2, 3]. Following metabolic activation, VC is able to bind covalently to nucleic acid bases. Four VC–DNA adducts have been identified *in vitro* and *in vivo:* 7-(2-oxoethyl)guanine which is the major VC–DNA adduct, and the three cyclic adducts, 1,N^6-ethenoadenine, 3,N^4-ethenocytosine and N^2,3-ethenoguanine [4] (Fig. 1). The formation of 3,N^4-ethenodeoxycytidine (εdCyd) and tentatively of 1,N^6-ethenodeoxyadenosine (εdAdo) in liver DNA from rats exposed to VC for 2 years was reported in 1978 by Green and Hathway [5]. However, subsequent analyses of DNA from rodents, following a short exposure to [^{14}C]VC, failed to show the formation of these two adducts [6]. It is only recently that Eberle *et al.* [7], using specific monoclonal antibodies together with sensitive immunoanalytical methods, confirmed the formation of εdCyd and εdAdo in DNA after short exposure of rats to VC. In the work presented here, we have investigated further the formation of these etheno adducts to determine whether it can be related to the organotropism and the age-related sensitivity of VC-induced carcinogenesis [8].

MATERIALS AND METHODS

Chemicals. Vinyl chloride (3% in N_2, purity 99.9%) was obtained from Messer Griesheim (F.R.G.). Nucleosides and bases were purchased from P.L. Biochemicals (St Goar, F.R.G.) or Sigma Chemical Co. (St Louis, MO).

Enzymes were obtained from Boehringer Mannheim (Mannheim, F.R.G.).

[^{3}H]εdAdo (sp. act. 26 Ci/mmol) and [^{3}H]εdCyd (sp. act. 18 Ci/mmol) were prepared as previously described [7].

Animals. BD VI rats were bred in this laboratory and were given commercial rat chow (Extra Labo from Société Piétremont, Provins, France) and water *ad lib.*

VC exposure. A dynamically operating inhalation exposure system was used according to the original design from Barrow and Steinhagen [9], with some modifications. It consisted of an acrylic plastic chamber (68 × 52 × 52 cm) divided into two compartments which could accommodate up to four normal rat cages. The chamber inlet and outlet were fabricated from 4 cm O.D., high-density polyethylene tubing. VC was trapped downstream of the chamber in a system which consisted of four 0.25 m O.D. × 1.23 m high-density polyethylene cylinders connected in series. The first two cylinders were filled with Siliporite NK 30 (CECA SA, Velizy-Villacoublay, France) and the last two with activated charcoal (CECA). A rotary vane vacuum pump fitted with a T connection and a ball valve at the intake

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‖ Abbreviations: VC, vinyl chloride; εdAdo, 1,N^6-ethenodeoxyadenosine; εdCyd, 3,N^4-ethenodeoxycytidine; dAdo, 2′-deoxyadenosine; dCyd, 2′-deoxycytidine; dGuo, 2′-deoxyguanosine.

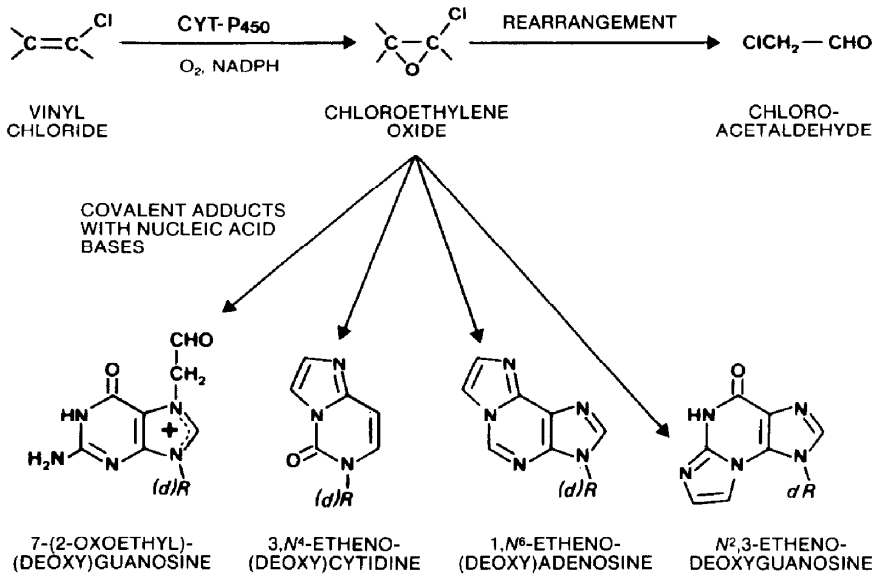

Fig. 1. Vinyl chloride metabolites and known nucleic acid adducts.

was used to draw the test atmosphere through the chamber and the traps. Chamber airflow was adjusted by means of the ball valve to correspond to about 15 chamber volume changes per hour. Under these conditions, the chamber was operated at a sub-atmospheric pressure, so that system leakage was minimized. Diluted VC (3% in nitrogen) was metered from a pressurized cylinder fitted with a fine-adjustment valve and a calibrated flowmeter, and was mixed with ambient air inside the chamber inlet pipe. Samples of the chamber atmosphere were taken through sampling ports and the concentration of VC was determined by gas chromatography on Porapack QS (80–100 mesh, from Waters Associates, Milford, MA, U.S.A.), with flame ionization detection.

During exposure, rats were housed in four plastic and brass cages placed in the inhalation chamber and had access to food and water *ad lib.* Group I consisted of 39 seven-day-old male and female rats, originating from six litters; they were kept with their mothers during exposure. Group II consisted of 10 13-week-old male rats.

Animals from the two groups were exposed for 14 consecutive days (7 hours/day) to 500 ppm VC.

Animals were killed either immediately after the end of exposure (group II) or 12 hr later (group I). Organs (liver, lungs, brain and kidneys) were removed and kept at −80° for further processing.

Isolation of DNA. DNA was prepared according to the method described by Krieg *et al.* [10]. Deep-frozen tissue samples were homogenized using a mechanical device, the 6700 Freezer/Mill (Spex Industries Inc., Edison, NJ, U.S.A.).

The resulting frozen powder (1 g) was then immediately transferred into a mixture of 10 mL of 0.3 M sodium acetate pH 7.2, 0.5% (v/v) SDS, 5 mM EDTA and of 10 mL of 66% (v/v) phenol in 0.3 M sodium acetate pH 7.2, 0.1% hydroxyquinoline (equilibrated overnight) and gently shaken by inversion for 20 min. The resulting solution was then extracted successively with chloroform: isoamyl alcohol (25:1 v/v) and chloroform, and nucleic acids were precipitated from the final aqueous phase by addition of two volumes of cold ethanol. Contaminating RNA was eliminated by a 1-hr treatment of 37° with bovine pancreas RNase (100 μg/mL, pre-heated at 80° for 30 min) in 1 mM EDTA, 10 mM Tris–HCl buffer (pH 7.4).

DNA was enzymatically hydrolysed using DNase I (50 μg/mg DNA, 30 min, 37°), snake venom phosphodiesterase and alkaline phosphatase (15 μg and 10 units/mg DNA, respectively, 2 hr, 37°) in a 0.05 M Tris–HCl buffer (pH 6.75) containing 0.01 M $MgCl_2$.

High-performance liquid chromatography. Chromatographic runs were performed on a Waters Associates HPLC system consisting of two Model 510 pumps, an Automated Gradient Controller and a Model U6K universal injector.

DNA hydrolysates were separated at room temperature on a 10 mm × 25 cm Nucleosil C18 5 μm reverse phase column (Société Française Chromato Colonne, Gagny, France) using a gradient elution previously described [11] with the following modifications: step 5, 13 to 25% eluant B (linear 20 min); step 6, 25% eluant B (isocratic 15 min). The flow rate was set at 3 mL/min.

The eluate was monitored at 254 nm using a Perkin-Elmer LC-15 UV detector and fractions containing individual 2′-deoxynucleosides were pooled. Amounts were determined by absorbance at 260 nm using the following extinction coefficients ($\times 10^{-3}$) thymidine, 8.75; dCyd, 7.35; dAdo, 14.9 and dGuo, 11.75.

Retention times for etheno adducts were determined with ^{3}H-labelled εdCyd and εdAdo and were,

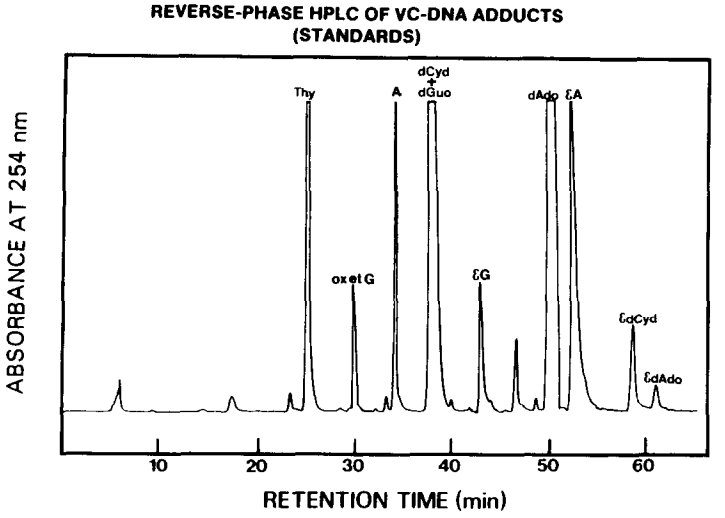

Fig. 2. Reversed-phase HPLC of VC–DNA adducts (standards). Thy, thymidine; oxetG, 7-(2-oxoethyl)-guanine; A, adenine; dCyd, 2′-deoxycytidine; dGuo, 2′-deoxyguanosine; εG, N^2,3-ethenoguanine; dAdo, 2′-deoxyadenosine; εA, 1,N^6-ethenoadenine; εdCyd, 3,N^4-etheno-2′-deoxycytidine; εdAdo, 1,N^6-etheno-2′-deoxyadenosine.

respectively, 57.75 and 60.20 min. The corresponding fractions were pooled, freeze-dried and reconstituted in a small volume of radioimmunoassay buffer.

Detection of 1,N^6-*ethenodeoxyadenosine and* 3,N^4-*ethenodeoxycytidine.* εdAdo and εdCyd were determined in the respective fractions by competitive radioimmunoassay as previously described [7, 12, 13] using monoclonal antibodies EM-A1 and EM-C1, respectively, and [^{3}H]εdAdo and [^{3}H]εdCyd as tracers. The values were read from a standard curve and converted to femtomoles of εdAdo or εdCyd.

RESULTS AND DISCUSSION

In rats from group I (young BD VI rats exposed to 500 ppm VC for two weeks), DNA adducts were determined in several organs 12 hr after the end of VC exposure. εdAdo and εdCyd were measured after enzymatic DNA hydrolysis using HPLC prepurification and competitive radioimmunoassay determination. The reproducibility of the analysis was assessed using DNA modified *in vitro* with chloroethylene oxide. Standard deviation was ±15% for εdAdo analysis (N = 27) and ± 38% for εdCyd analysis (N = 15); N represents the number of independent analyses (including DNA hydrolysis, HPLC separation and RIA determination) from the same modified DNA sample. Under the HPLC conditions, εdCyd and εdAdo were well separated from other VC–DNA adducts and from natural 2′-deoxyribonucleosides, as shown in Fig. 2. Between 4 and 7 mg of DNA were analysed for each organ (approx. 2 mg per chromatographic run) and fractions containing εdAdo and εdCyd were collected from several chromatographic runs and pooled for radioimmunoassay determination, using monoclonal antibodies EM-A1 and EM-C1 [7]. Both εdAdo and εdCyd were detected in the liver, lungs and brain of VC-treated young BD VI rats. The results (Table 1) when expressed as the molar ratios of the respective etheno adduct to the corresponding unmodified 2′-deoxyribonucleoside ranged from (0.6 to 1.3) × 10^{-7} for εdAdo/dAdo and from (2.05 to 4.8) × 10^{-7} for εdCyd/dCyd. Moreover, the ratios between the levels of modification of the two adducts were the same in these three organs: for each of them, there was three times as much εdCyd as εdAdo. In contrast, no DNA ethenobase adducts were detected in kidneys from the same young rats nor in organs from untreated young BD VI rats, at a detection

Table 1. VC–DNA adducts in newborn* and adult† BD VI rats exposed to VC

Organ‡	Molar ratio (× 10^{-7}) of εdAdo/dAdo	εdCyd/dCyd
Liver (newborn)	1.30§	4.92§
	1.31	4.67
Liver (adult)	0.19	0.80
Lung (newborn)	1.05§	2.46§
	0.89	2.22
Brain (newborn)	0.60	2.16§
		1.95
Kidney (newborn)	ND	ND

* Seven-day-old rats exposed to 500 ppm VC in air for 14 consecutive days, 7 hr per day; killed 12 hr after the end of exposure.

† Thirteen-week-old rats exposed to 500 ppm VC in air for 14 consecutive days, 7 hr per day; killed immediately following the end of exposure.

‡ Organs were pooled for DNA analyses.

§ Values from two analyses are listed.

ND, not detected (detection limit: 3 × 10^{-8} for εdCyd/dCyd and 2.4 × 10^{-8} for εdAdo/dAdo).

limit of 3×10^{-8} for εdCyd/dCyd and 2.4×10^{-8} for εdAdo/dAdo. In rats from group II (adult BD VI rats exposed to 500 ppm VC for 2 weeks), εdAdo and εdCyd were measured in liver only, requiring the pre-purification of 50 mg of DNA. The molar ratios (Table 1) were 0.19×10^{-7} for εdAdo/dAdo and 0.80×10^{-7} for εdCyd/dCyd.

Thus the use of specific monoclonal antibodies previously described and characterized [7] allowed the detection of εdCyd and εdAdo in several target organs of VC-exposed rodents. The results reported here, confirming those obtained by Eberle *et al.* [7], clearly demonstrate the formation of these two etheno adducts in DNA *in vivo*. The miscoding potential of these ethenobase adducts has been reasonably well demonstrated, suggesting that they might play an important role in the initiation step of VC-induced tumorigenesis, through induction of point mutations in DNA [14–17].

Maltoni *et al.* (Ref. 8; experiment BT 14) found that newborn animals as compared to adults are much more sensitive to the hepatocarcinogenic action of VC. After 5 weeks exposure to 10,000 ppm VC (from day 1 after birth), newborn animals showed an increased incidence of liver tumours as compared to adult rats, i.e. 34.1% versus 0.8% angiosarcomas and 45.5% versus 0.8% hepatocellular carcinomas. Based on these data, Laib *et al.* [18] measured the induction of preneoplastic foci in newborn and adult rats exposed to VC and defined a period in the early life (between days 7 and 21 after birth) where the animals were most sensitive to VC. Moreover, after exposing rats to [1,2-^{14}C]VC, the same authors found that the amount of 7-(2-oxoethyl)guanine formed in liver DNA of young Wistar rats was five times that in adults [19]. In the work presented here, we have measured εdAdo and εdCyd in DNA of several organs of 21-day-old and 13-week-old BD VI rats exposed to VC. In liver DNA of young animals, about six times as much of each adduct was formed as compared to adult rats. Thus, our results on DNA modification well reflect the higher sensitivity of young animals as compared to adults to the hepatocarcinogenic action of VC, possibly due to the high cellular proliferation in the liver of young animals, yielding more DNA accessible to the reactive metabolites of VC and a higher rate of induced mutations as a result of unrepaired VC-DNA adducts.

Extensive studies done by Maltoni *et al.* [8] have shown that liver is the main target organ in VC-induced carcinogenesis. In the present study we also found that liver is the organ with the highest level of modification, this certainly reflects well the ability of this organ to activate VC via microsomal-cytochrome P-450 dependent monooxygenases (Fig. 1) [20]. Although we have no carcinogenicity data for BD VI rats, VC has been shown to induce liver tumours in four other rat strains [8, 21–23] and thus it is reasonable to assume that BD VI rats would not be resistant to the carcinogenic action of VC. However, VC is a versatile carcinogen which induces tumours at different organ sites. In addition to liver DNA, we have also detected εdAdo and εdCyd in DNA from lungs. Indeed Maltoni *et al.* (Ref. 8; experiment BT4001 and 4006) have shown that perinatal exposure to VC was a crucial factor to enhance the neoplastic response of lung: after exposure of female Sprague–Dawley rats from day 12 of pregnancy during 15 or 76 weeks, there was an increased incidence of lung adenomas in the offspring. We have also detected εdAdo and εdCyd in brain DNA, which is also a known target organ for VC-induced carcinogenesis. In the above experiment by Maltoni *et al.* [8] an increased incidence of neuroblastoma in both parents and offspring was observed as compared to untreated animals. In contrast, in this experiment, no increased incidence of nephroblastoma was observed. A significant increase in kidney tumours had been found only following long-term exposure to VC [8]. In the present study, we have not been able to detect etheno adducts in kidney DNA after short-term exposure, possibly indicating the lower capacity of this organ to metabolize VC [20]. Other organs, such as mammary glands and zymbal glands, are also targets in VC-induced carcinogenesis, but the detection of etheno adducts in these glands is restricted by the large amounts of DNA which are needed for the analysis by our technique. In conclusion, our data although limited by the number of organs analysed are in agreement with the known organotropism of VC-induced carcinogenicity; the levels of etheno adducts in DNA was found to be a good indication of the tissues which are at risk for tumour development.

The method reported here also permits the kinetics of formation and repair of ethenobases in DNA *in vivo* to be determined; these studies are in progress.

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