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The rat liver foci bioassay: II. Investigations on the dose-dependent induction of ATPase-deficient foci by vinyl chloride at very low doses

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In order to study the dose-dependence of the genotoxic effect of vinyl chloride (VC) hepatocellular ATPase-deficient foci were evaluated after subchronic exposure of newborn rats. Wistar rats were exposed from day 1 after birth over 10 weeks to 10, 40, 70, 150, 500 and 2000 p.p.m. VC (8 h/day; 5 days/week). One week after cessation of exposure hepatic ATPase-deficient foci were quantitated. For a subsequent investigation lower dose range groups of female and male Wistar and Sprague-Dawley rats were exposed (8 h/day; 5 days/week) to 2.5, 5, 10, 20, 40 and 80 p.p.m. VC. Exposure started at day 3 of life and lasted for 3 weeks. After cessation of exposure the animals were maintained for 10 weeks without further treatment until ATPase-deficient foci were quantitated. Both sets of experiments revealed a straight linear relationship between the dose of VC and the % foci area induced. Within the dose range investigated, no obvious threshold for the induction of pre-neoplastic foci by VC was observed.

Introduction

One major point of controversy in risk assessment of chemical carcinogens and in basic research on mechanisms of chemical carcinogenesis is that of 'threshold' doses. Detoxification of reactive metabolites and DNA-repair processes have been advoked to argue in favour of 'thresholds'. On the other hand, DNA binding studies with established carcinogens so far have revealed linear dose-responses over wide dose ranges (1). In dose ranges covered by long-term bioassays the action of 'genotoxic' carcinogens is found irreversible (persistent) and additive (2–5). For statistical reasons in long-term animal experiments (20–200 animals per group) tumor incidences of 5–10% over controls can be detected with statistical significance. Valid dose-response data at ~1% incidence would require ~500 animals per group; thus the animal number becomes a very limiting factor.

Gehring and co-workers (6) have used combined bioassay (7) and metabolic (8) data on vinyl chloride (VC)* for risk prediction at low doses. They argued that a threshold for the carcinogenicity of VC might exist below exposure concentrations of 50 p.p.m. because of the lacking depletion of hepatic glutathione in this dose range (8).

The aim of the present study was to investigate the dose-dependence of carcinogenicity of VC over an extended dose range, including very low exposure concentrations (2.5 p.p.m.). As a sensitive parameter of carcinogenicity the development of pre-neoplastic enzyme-altered foci was investigated. Adenosinetriphosphatase (ATPase)-deficient foci were quantitated as an endpoint of the 'rat liver foci bioassay'. As foci area follows dose-time response relationships identical to that observed for the induction of liver tumors (4) this parameter was evaluated to describe the dose-response relationships observed.

*Abbreviations: VC, vinyl chloride; ATPase, adenosine-5'-triphosphatase.

Materials and methods

Animals

Pregnant Wistar and Sprague-Dawley rats (Ivanovas Kissleg, FRG) were obtained 7–5 days before parturition and housed singly. They received a standard pellet diet (Altromin 1320, Lage, FRG) and drinking water *ad libitum*.

Protocols

Exposure schedule A. Groups of 4–5 male and female newborn Wistar rats (randomly assigned from four individual litters to two different exposure groups) were exposed together with the mother animals, starting from day 1 after birth for 10 weeks (8 h/day, 5 days/week) to 10, 40, 70, 150, 500 and 2000 p.p.m. VC. At the end of exposure the individual exposure groups were maintained for 1 week without further treatment until ATPase-deficient foci were quantitated.

Exposure schedule B (see Figure 1). Newborn male and female Wistar and Sprague-Dawley rats were randomly assigned from four individual litters to two different exposure groups and exposed to VC together with the mother animals. Exposure started at day 3 of life and lasted for 3 weeks (8 h/day; 5 days/week). Exposure concentrations were 2.5, 5, 10, 20, 40 and 80 p.p.m. VC. At the end of exposure the animals were maintained for 10 weeks without further treatment until ATPase-deficient foci were quantitated. Animals of each strain and sex which were exposed to air only served as controls.

Exposure of the animals to VC (chemical purity 99%, from Linde, Unterschleissheim, FRG) was carried out as already described (9). Exposure concentration was routinely controlled by g.l.c. Exposure to high concentrations of VC (2000, 500, 150 p.p.m.) was constant with daily deviations of ±5% of the intended value.

At the lower concentrations, mean values for the 3 week exposure periods with maximal daily deviations were 80 ± 11 p.p.m., 41 ± 6 p.p.m., 20 ± 5 p.p.m., 5.5 ± 2 p.p.m. and 2.5 ± 0.8 p.p.m.

In the dose range where first order kinetics apply (10) the metabolized amount of VC (dose) is a function of the product of concentration and time and independent of the individual exposure profile. This implies that relatively

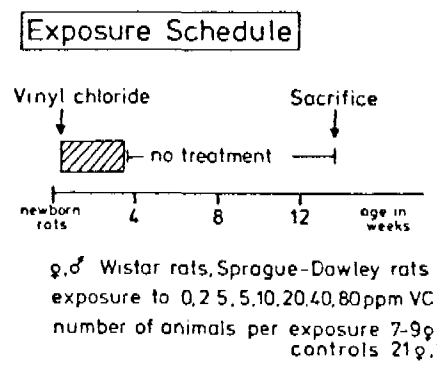

Fig. 1. Schematic representation of the exposure schedule (schedule B) applied: exposure of 3-day-old Wistar and Sprague-Dawley rats of both sexes (8 h/day; 5 day/week) to VC for 3 weeks. After cessation of exposure the animals were maintained without further treatment until ATPase-deficient foci were quantitated.

short deviations of the exposure concentration from a mean can be tolerated because the mean exposure concentration is of relevance only for such studies.

Histochemistry

The animals were sacrificed and cryostat sections from two liver lobes of each animal were prepared. The sections were stained for ATPase according to Wachstein *et al.* (11). Foci area was quantitated in five cryostat sections per liver (~10 cm²) using the Zeiss lattice plate II-100/25. Foci area was expressed as % of liver area. The number of animals evaluated in each individual dose group is included in the legend to the corresponding figures. Results for each exposure group are presented as means ± standard deviation. Dose-response curves are calculated by linear least squares fittings

Results

In a preliminary experiment groups of 4–5 male and female Wistar rats were exposed to concentrations between 10 and 2000 p.p.m. VC according to schedule A and ATPase-deficient foci were quantitated in the livers of the animals 1 week after cessation of exposure.

Figure 2 compares the influence on foci formation of saturation of VC metabolism with that on formation of angiosarcoma (7) evaluated by Gehring *et al.* (16). In female and male (data not shown) rats the foci areas show a linear increase with increasing exposure concentration from 10 p.p.m. up to 500 p.p.m. VC where they reach a plateau of response.

For a more detailed investigation in the lower dose range two strains of rats, a higher number of animals per individual dose group and a much higher number of controls was used. The animals were treated according to schedule B (Figure 1) and ATPase-deficient foci were quantitated 10 weeks after cessation of exposure (9). In Figures 3 and 4 foci area in the livers of male and female Wistar and Sprague-Dawley rats induced by VC is plotted versus the different exposure concentrations. To demonstrate the large interindividual differences in susceptibilities of the animals the standard deviations of the individual exposure groups are also included in the figures. (In this investigation the individual standard errors of estimate are ~1/3 of the standard deviations.)

Figures 3 and 4 show that for male and female rats of both strains the foci area induced increases in proportion to the exposure concentration (dose) applied. These dose-response relationships thus established within the range of 2.5 to 80 p.p.m. can be described, within the limits of error of the method, by linear dose-response curves which run through the origin.

A comparison of the dose-dependent increase of foci area in both rat strains and sexes reveals increases in susceptibilities in terms of induction of hepatocellular foci in the following order: male Sprague-Dawley rats < male Wistar rats < female Sprague-Dawley rats < female Wistar rats. Also, it must be noted that mean foci area induced by the two lowest doses of VC in male rats of both strains lies within the range of foci area of the corresponding controls.

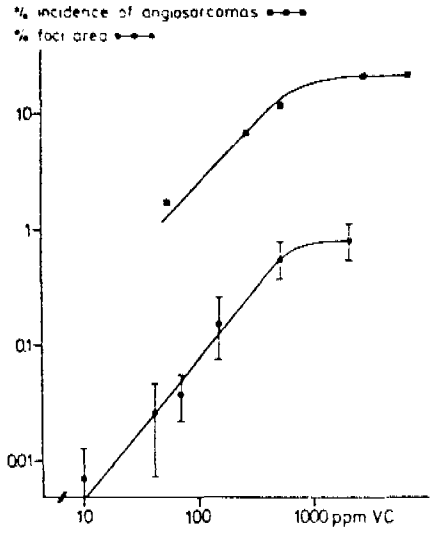

Fig. 2. Incidence of liver angiosarcomas (7) (top) and ATPase-deficient foci induced in female Wistar rats (bottom) plotted versus exposure concentration (log/log scale) Incidence of angiosarcomas (6) and % foci area increase linearly with increasing exposure concentration until a plateau is reached.

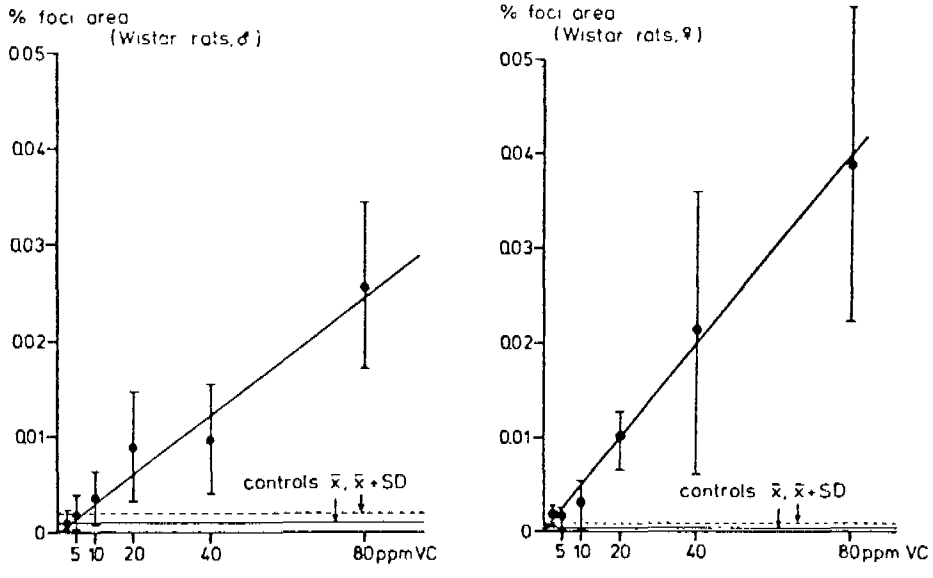

Fig. 3. Dose-dependent induction of hepatocellular ATPase-deficient foci by VC in male (left) and female (right) Wistar rats. The dose-response relationships can be described within the accuracy of the method by linear dose-response curves which run through the origin. Number of animals (in parenthesis) within the individual dose groups examined for males: controls (14), 2.5 (8); 5 (6); 10 (7); 20 (5); 40 (10), 80 (10); and for females: controls (20), 2.5 (8); 5 (9); 10 (4); 20 (6); 40 (12), 80 (11) Dose-response relationship for males is described by the linear curve $y = 7.4 \times 10^{-4} + 0.32 \times 10^{-3} x$ with a coefficient of correlation of 0.86, and for females $y = 1.8 \times 10^{-4} + 0.5 \times 10^{-3} x$, with a coefficient of correlation of 0.81

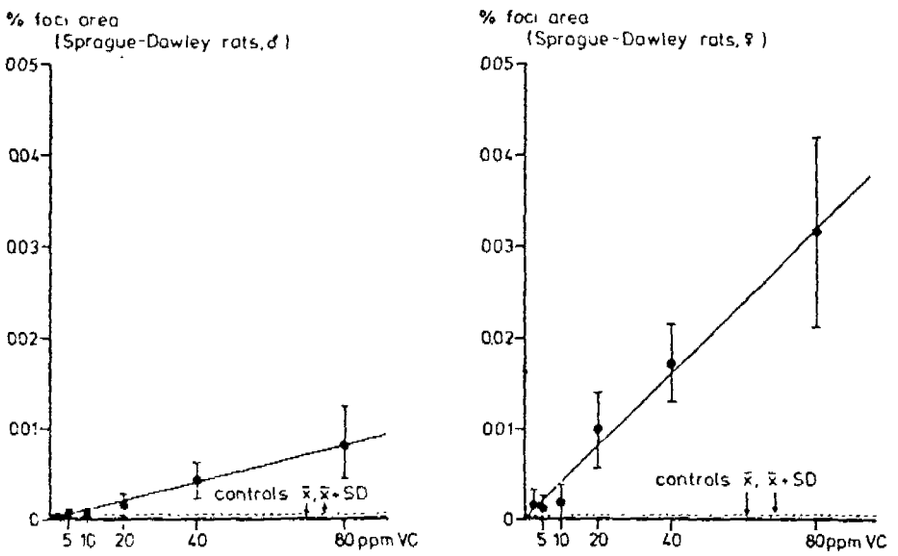

Fig. 4. Dose-dependent induction of hepatocellular ATPase-deficient foci by VC in male (left) and female (right) Sprague-Dawley rats. The dose-response relationship can be described within the accuracy of the method by linear dose response curves which run through the origin. Number of animals (in parenthesis) within the individual dose groups examined for males: controls (27); 2.5 (6); 5 (2); 10 (8); 20 (5); 40 (4); 80 (10); and for females: controls (10), 5 (15); 5 (7), 10 (9); 20 (7), 40 (7), 80 (11). Dose-response relationship for males is described by the linear curve $y = 3.5 \times 10^{-4} + 0.11 \times 10^{-3} x$ with a coefficient of correlation of 0.80 and for females $y = 0.70 \times 10^{-4} + 0.40 \times 10^{-3} x$, with a coefficient of correlation of 0.91.

Discussion

The results of both studies (schedule A and schedule B) clearly demonstrate a linear relationship between the dose (concentration) of VC applied and the area of ATPase-deficient foci induced, which ranges in doses over three orders of magnitude (2.5 – 500 p.p.m. VC).

It is now clear that pharmacokinetics of a chemical have to be considered for risk evaluation (2,5). The risk of tumor development from VC is not directly related to exposure concentration but to the rate of metabolism of VC which is saturable at high exposure concentration (6). As VC metabolism follows first order kinetics until saturation occurs at high doses, tumor incidences increase linearly with increasing exposure concentration until they reach a plateau at ~500 p.p.m. This has been demonstrated for angiosarcoma incidence in rats by Gehring *et al.* (6) evaluating bioassay data on VC (7, see Figure 2).

Our results show that the induction of ATPase-deficient foci in female (see Figure 2, bottom) and male (results not shown) Wistar rats is in accordance with these findings. The dose-response relationships thus established for Wistar and Sprague-Dawley rats of both sexes in the lower dose region (2.5 – 80 p.p.m. VC) can be described by linear dose-response curves which run through the origin within the accuracy of the method.

This exemplifies that within the dose range investigated no 'threshold' dose exists for VC below which the response probability is more than proportionally decreased. Earlier investigations on covalent binding of [^{14}C]VC to cellular macromolecules are supportive of this view (12). In addition, the linearity of the dose-response curves disproves the hypothesis that a 'threshold' for the carcinogenic action of VC in rats may exist at exposure concentrations below 50 p.p.m. (5, 8).

These investigations also demonstrate that with the appropriate protocol (9), the 'rat liver foci bioassay' provides a very sensitive tool for detection of carcinogenicity. By quantitation of ATPase-deficient foci in the liver of rats, dose-response relationships can be determined down to such low dose ranges where the carcinogenic risk caused by the substance can no longer be distinguished from that of non-exposed control animals.

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