

Quantitative Aspects of Chemical Carcinogenesis and Tumor Promotion in Liver

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Chronic exposure of rodents to high dose levels of drugs, food additives and environmental chemicals frequently results in liver enlargement. Several of these compounds have been found to enhance the incidence of liver tumors in animals briefly exposed previously to hepatocarcinogens. Accordingly, it has been advanced that these agents act as tumor promoters. This contention has remained subject of controversy following reports that these substances may also cause liver tumors in non-carcinogen-treated rodents, particularly in those characterized by a relatively high incidence of "spontaneous" liver tumors. Since many of these chemicals are in common use, a crucial question would seem to be whether such effects are due to facilitation of the expression of pre-existing oncogenic potential, i.e., to tumor promotion, or to the synergistic action of weakly carcinogenic agents. As a result of mechanistic differences tumor promotion and syn-carcinogenesis must exhibit different dose-time-response characteristics, and, accordingly, it should be possible, in principle, to discriminate between these phenomena. However, since tumor manifestation periods in low-dose groups frequently exceed the animals average lifespan, this approach may not always yield conclusive data, unless a sensitive early marker of carcinogenic activity can be employed. There is evidence that enzyme-deficient preneoplastic areas in liver can be used for this purpose. A strong quantitative correlation between carcinogen dose, the extent of ATPase deficient areas, and the subsequent appearance of tumors has now been established for a number of hepatocarcinogens. Experimental data are consistent with the concept that two critical events (hits) are required for induction of ATPase deficiency in hepatocytes. The first hit is carcinogen-dependent, whereas the second hit would seem to be due to time-dependent event(s). Tumor-promoters, such as phenobarbital, were found to accelerate and increase formation of preneoplastic islets. This evidence, together with data indicating that the compound is devoid of carcinogenic potential, suggests that phenobarbital may be operative at relatively early stages of hepatocarcinogenesis by increasing the probability of the occurrence of the time-dependent second hit. Such effects are dose-dependent and appear to be related to the induction of liver enlargement. The changes in hepatocellular ploidy status and atypical nuclear figures observed during phenobarbital treatment and cessation thereof, suggest that this compound might induce abnormal redistributions of genetic material. It is postulated that these cytological changes may result in phenotypical manifestation of recessive oncogenic information.

Introduction

Ever since the discovery that mouse skin carcinogenesis can be enhanced by chronic application of the skin irritant croton oil (1) and the subsequent isolation and characterization of phorbol esters as active components (2), tumor promoters have proved to be useful model compounds in studies on the nature of critical steps in chemical carcinogene-

sis (3). In the case of tumor promotion in liver, however, investigations have not been focused primarily on the elucidation of stages in the carcinogenic process, but were more concerned with the assessment of the toxicological significance of tumor-promoting drugs and environmental chemicals. These investigations were initiated by the observation that exposure of animals to high doses of drugs frequently resulted in pronounced liver enlargement (4-6), and the general significance of this phenomenon is illustrated by a report showing that liver enlargement may occur in approximately 75% of chronic feeding studies with newly introduced

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formation in dieldrin-treated CF-1 mice (33) exhibits a dose time-response relationship strikingly similar to that seen with TPA. However, that this approach may not always yield conclusive data, since tumor manifestation periods in low dose groups may frequently exceed the animal's average lifespan. This would appear to be a general characteristic of drugs with controversial tumorigenic properties, such as dieldrin or DDT. This problem could be overcome, however, by employment of a sensitive early marker of carcinogenic activity.

Preneoplastic Areas as Quantitative Early Markers of Liver Carcinogenesis

In recent years, evidence has accumulated, that in the case of liver, putatively preneoplastic areas can serve this purpose (34-40). These areas, commonly called islets, are deficient in enzymes such as ATPase or glucose-6-phosphatase, and may also show glycogen storage or a positive γ -glutamyl transferase reaction, as well as considerably enhanced epoxide hydrolase levels (41). It is not clear at present whether these cellular alterations are caused by somatic mutations, and, if so, whether these mutations are directly related to the carcinogenic process. There is, however, a strong quantitative correlation between carcinogen dose, the extent of enzyme-deficient areas, and the subsequent appearance of liver tumors (10, 35, 42). The development of an arbitrarily defined extent of ATPase-deficient areas in livers of rats continuously treated with various levels of diethylnitrosamine was found to show a dose-time-response relationship with characteristics identical to that observed for the induction of liver tumors (Fig. 4). This quantitative correlation has recently been confirmed for *N*-nitrosomorpholine (Fig. 4). These studies have also provided evidence for the actual number of critical events, also referred to as hits, that must occur before ATPase deficiency becomes phenotypically manifest. To this end, a mathematical model was developed which takes into account parameters such as the number of susceptible liver cells, the number of critical genes per cell, the risk per unit carcinogen dose for an intact critical gene to be hit and the minimum number of hits required for induction of islet cells (43). A comparison of the expected curves for varying numbers of hits with the actual number of islets observed after exposure of rats to increasing total doses of diethylnitrosamine (constant dose level administered for periods up to 6 weeks) revealed that experimental results are consistent with the assumption that a minimum of two hits is

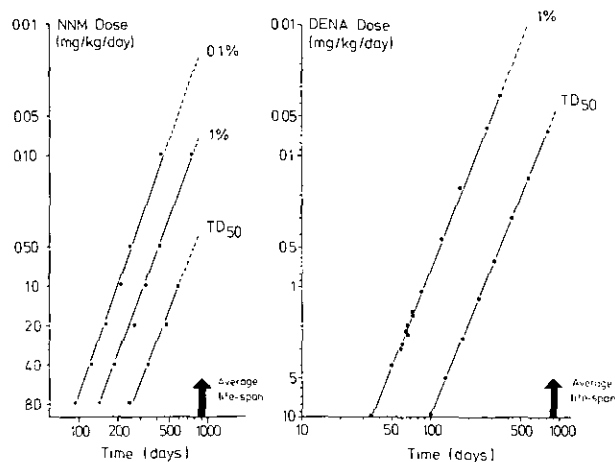


FIGURE 4. Dose time-response relationship for the induction of defined volumes of preneoplastic islets in rat liver by *N*-nitrosomorpholine (NNM) and by diethylnitrosamine (DENA) and its quantitative correlation with the induction of liver tumors. Adult female Lewis rats were continuously exposed to the indicated levels of NNM and DENA. For every treatment group, the exposure time required for the induction of 0.1 and 1.0% preneoplastic areas was determined on the basis of quantitative analyses of the preneoplastic response in a total of 18 animals, killed at different time intervals. These time periods were recorded versus dose in a double logarithmic plot and compared with the dose-time-response relationship observed for the induction of 50% liver tumor mortality (TD_{50}) (based on observations in 12 animals/treatment). The parallel lines indicate an identical dose-time-response dependency, i.e., $(\text{dose})(\text{time})^n = \text{constant}$.

required for the expression of the preneoplastic cell phenotype (Fig. 5). Results in support of this concept have been obtained from chronic treatment studies with *N*-nitrosomorpholine at various levels of exposure. The assumption that two critical hits are required for enzyme deficiency is consistent with the observed time-response relationship for islet induction in *N*-nitrosomorpholine hepatocarcinogenesis (Fig. 6). These data indicate that islet number is related to exposure time by a power of 2. In contrast, dose-islet number relationships show a slope characteristic of a one-hit mechanism. This latter phenomenon was also observed previously by Emmelot and Scherer (35). This evidence suggests that only the first hit is carcinogen-dependent, whereas the second hit, which leads to phenotypical manifestation of enzyme deficiency, would seem to be independent of further carcinogen treatment and caused by a time-dependent event.

Initiation and Promotion of Liver Carcinogenesis

These considerations explain the characteristics of islet formation following cessation of carcinogen

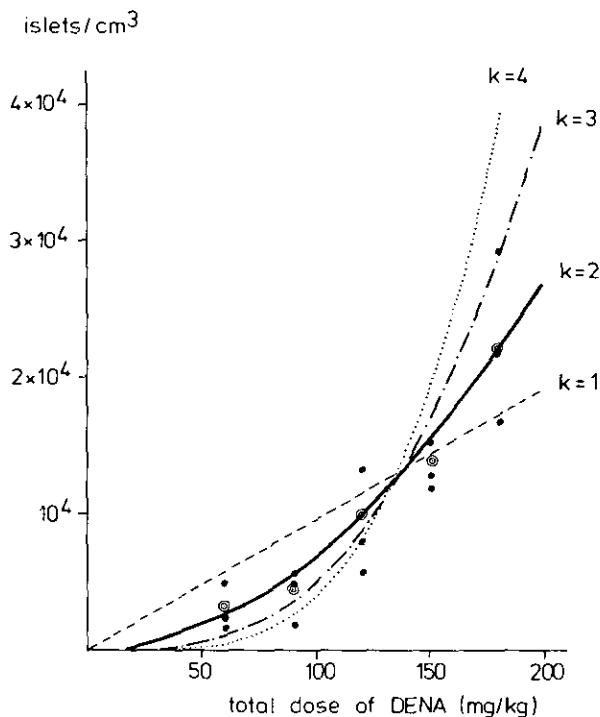


FIGURE 5. Multi-hit model for the induction of ATP-ase deficient cell clones in rat liver by carcinogens (43). The model equation is:

$$E(I/D) = N \left[\sum_{i=k}^n \binom{n}{i} (1 - e^{-\lambda D})^i (e^{-\lambda D})^{n-i} \right]$$

where $E(I, D)$ = expected number of ATP-ase deficient cell islets (I) per unit volume of liver tissue, at total carcinogen dose D , N = total number of susceptible liver cells/unit volume, k = minimum number of hits required for expression of islet cell phenotype, n = total number of ATP-ase relevant gene loci per liver cell, λ = probability per carcinogen dose unit for an intact ATPase-relevant gene locus to be hit. Expected curves were compared with experimental results obtained in rats exposed to 5 mg diethylnitrosamine (DENA)/kg BW/day for periods up to 6 weeks. Animals were killed and analyzed 6 weeks after the end of DENA treatment. Experimental results are consistent with the hypothesis that $k = 2$.

treatment, which is a necessary feature of initiation-promotion studies. Following discontinuation of diethylnitrosamine treatment, the number of preneoplastic islets continues to rise for a certain period of time, and after having reached a maximum gradually decreases until a steady-state level is established (Fig. 7). These characteristics of the preneoplastic response following cessation of carcinogen treatment have been observed by several groups and were interpreted in various ways. The finding that islet formation is initiated by a carcinogen-dependent first hit and requires a time-dependent second hit for its phenotypical manifestation may explain these characteristics on the premise that the

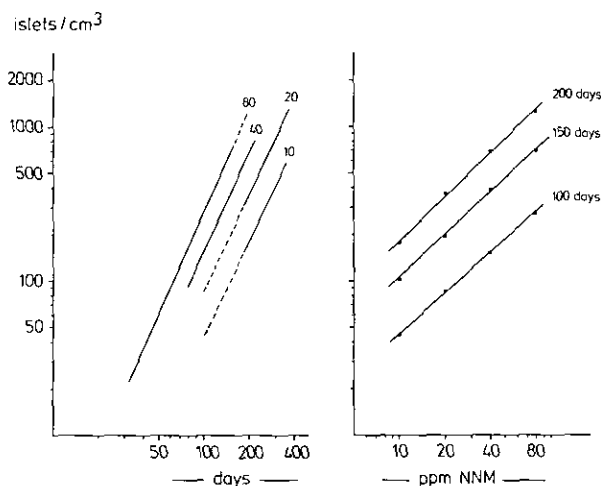


FIGURE 6. Dose-response and time-response kinetics for the induction of ATPase-deficient islets and liver in rat liver by *N*-nitrosomorpholine (NNM). Female adult Lewis rats were treated continuously with NNM at the dose levels of 10, 20, 40 and 80 ppm in drinking water (18 rats/treatment). The animals were killed sequentially (two to five animals at every time interval) and ATPase-deficient islets were morphometrically analyzed in three liver sections/animal: (—) observed (---) extrapolated, Regression analysis of relationship between islet number (y) and exposure time (x) yielded following equations: NNM 80 ppm: $\log y = -2.242 + 2.327 \log x$; NNM 40 ppm: $\log y = -1.554 + 1.865 \log x$; NNM 20 ppm: $\log y = -2.288 + 2.092 \log x$; NNM 10 ppm: $\log y = -2.251 + 1.947 \log x$.

genetic changes leading to ATPase deficiency are recessive in nature. Since the decrease in islet number usually starts after a time period similar to the reported average lifespan of hepatocytes, it is conceivable that enzyme-deficient cells are subject to physiological turnover unless they assume a more neo-plastic character. This turnover is likely to include hepatocytes with nonexpressed first hits, and this would explain the observation that no new islets are being formed in the steady-state situation (Fig. 7). Exposure to diethylnitrosamine-pretreated rats, to phenobarbital results in pronounced enhancement of the preneoplastic response (Fig. 8). During the initial stages of phenobarbital treatment, these effects were found to be due entirely to increases in the number of islets and not to any acceleration of islet cell growth. Similar to the changes observed in control animals, a maximum was reached, followed by a decline, until the number of islets remained constant. Both maximum and steady state were at considerably higher levels, however, and occurred at an earlier stage than in controls. This amplification of the preneoplastic response resulted in an early appearance of an increased number of islets with progressed neoplastic characteristics. This was indicated by the frequent

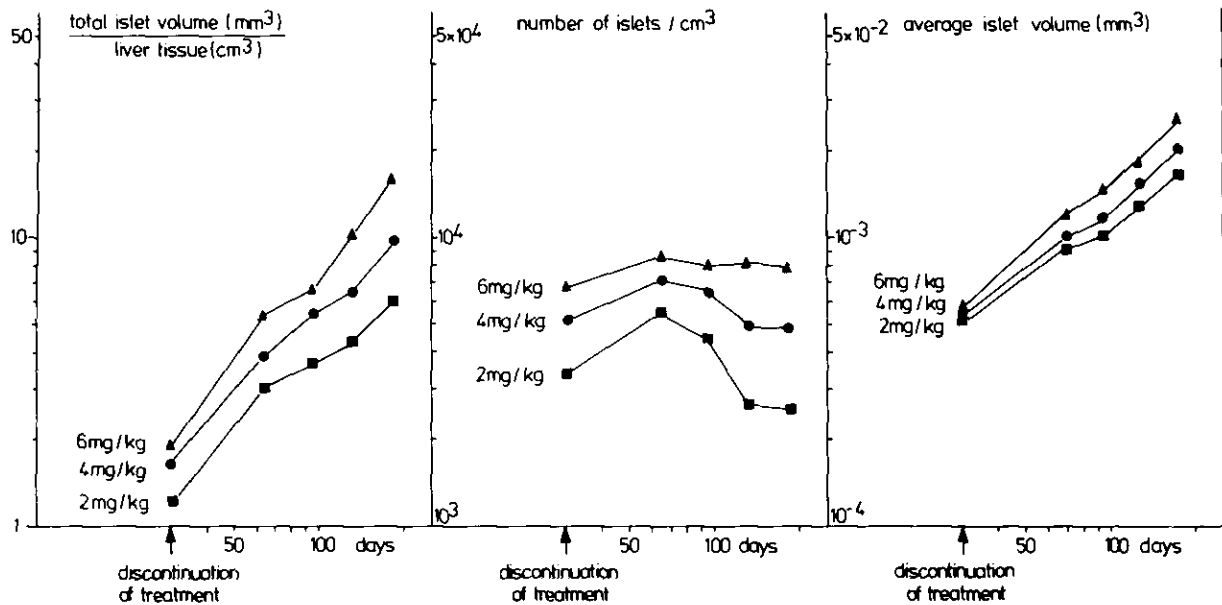


FIGURE 7. Changes in number, average and total size of ATPase-deficient islets in livers of Wistar rats following discontinuation of diethylnitrosamine (DENa) treatment. Animals were exposed to the carcinogen for a period of 4 weeks at indicated daily dose levels and killed sequentially (five animals/time interval) following discontinuation of DENa treatment.

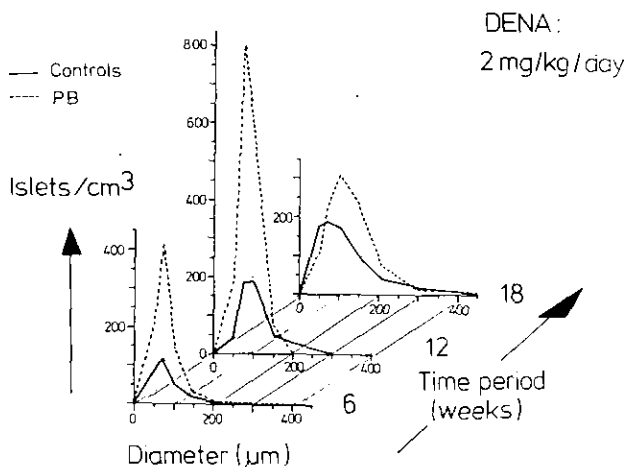


FIGURE 8. Effect of phenobarbital (PB) treatment on number and size distribution of ATPase-deficient islets in livers of Wistar rats pretreated with diethylnitrosamine (DENa): (—) controls; (---) PB-treated. Adult animals were pretreated with a daily dose of 2 mg DENa/kg BW for 4 weeks and subsequently treated or not with phenobarbital (dose equivalent to 75 mg/kg/day in drinking water). Groups of five animals were killed 6, 12 and 18 weeks after cessation of carcinogen pretreatment. Six sections per liver were morphometrically analyzed. Islet number per unit volume were estimated as described by Scherer et al. (45).

appearance of cellular phenotypes, such as intermediate and basophilic cells, that are characteristic of later stages of hepatocarcinogenesis (44). At this

time, islet size distribution in phenobarbital-treated rats showed a distinct shift to higher diameters. These results suggest that the observed enhancement of islet growth at later stages resulted from an accelerated expression of neoplastic character, and not from continuous stimulation of islet cell multiplication by the promoting drug.

The intensity of the effects of phenobarbital on the preneoplastic response was found to be dependent on the level of prior initiation. In the experiments shown in Figure 9, rats were pretreated with varying doses of diethylnitrosamine, followed or not followed by treatment with a constant dose of phenobarbital. Phenobarbital accelerates the formation of preneoplastic islets, and this suggests that the compound may be operative by amplifying the event leading to expression of the second time-dependent hit. This effect was most pronounced at the lowest level of exposure to the initiating carcinogen. Phenobarbital was increasingly ineffective the higher the level of prior initiation. This phenomenon is also illustrated in Figure 10, which includes effects observed with animals pretreated with 4 mg/kg diethylnitrosamine/day for 4 weeks. Similar results have been reported for the action of the classical tumor promoter TPA on DMBA-initiated skin carcinogenesis (47).

These results suggest that, as a general phenomenon, tumor promoters act by increasing the impact of the acceleration principle associated with time-dependent events in carcinogenesis.

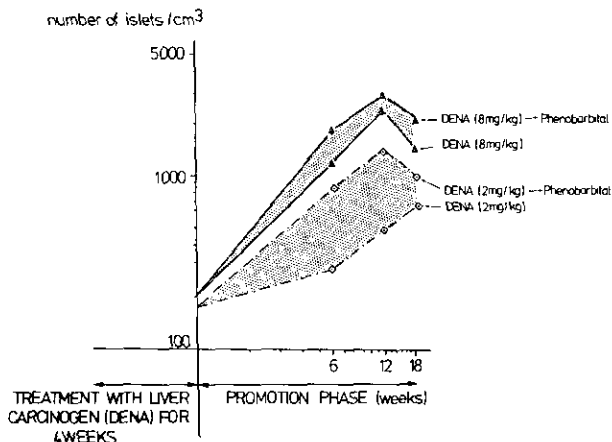


FIGURE 9. Effect of phenobarbital treatment on the number of ATPase-deficient islets in livers of rats pretreated with different levels of diethylnitrosamine. Experimental details as in Figure 8. The data observed in rats pretreated with 4 mg DENA/kg/day were omitted because of interference with the other curves. For the 4 mg DENA treatment group, control values were nearly identical to that seen in the treatment group DENA (2 mg) + phenobarbital. Results observed in the treatment group DENA (4 mg) + phenobarbital were slightly higher than those seen in the DENA (8 mg) controls.

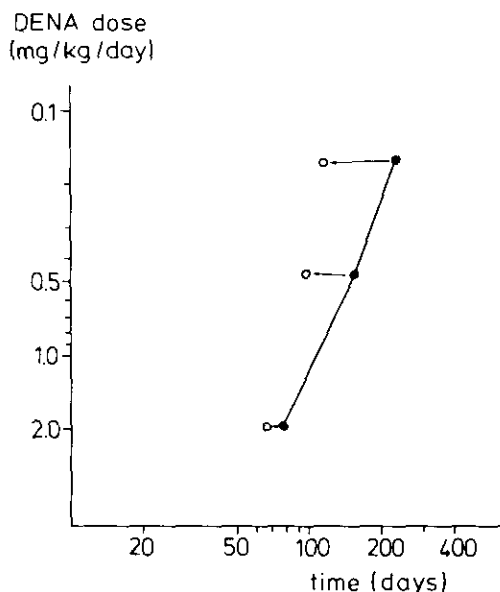


FIGURE 10. Effect of phenobarbital (PB) on diethylnitrosamine (DENA)-induced rat liver carcinogenesis: (●) DENA; (○) DENA + PB. Experimental details as in Figure 8. Carcinogenic response defined as the occurrence of 1% ATPase-deficient areas per unit liver volume. Effects of indicated daily DENA dose levels when given continuously correspond to those observed with the three levels of DENA pretreatment (2, 4 and 8 mg/kg/day for 4 weeks). The former dose levels are calculated from division of the total DENA dose administered by the number of days required for the development of 1% ATPase-deficient areas per unit liver volume (46).

Tumor Promotion in Liver: Mechanism, Dose Dependency and Toxicological Implications

Present evidence indicates that tumor promotion in liver by drugs is somehow correlated with organ enlargement. Effects on liver weight have been found to reach a plateau after a certain treatment period; in the case of phenobarbital this can usually be seen after 1 week of treatment. It follows that if proliferative processes are involved in tumor promotion, intermittent drug treatment should produce effects similar to those seen after continuous treatment. Accordingly, rats initiated with diethylnitrosamine were treated with phenobarbital for 1 week at 6-week intervals for a period of 18 weeks, and results were compared with those obtained with rats treated continuously (Fig. 11). Intermittent exposure to phenobarbital was found to result in similar, if not more pronounced increases in the number of preneoplastic islets in liver. The observation that a total phenobarbital dose of only 1/6 of that administered to continuously treated rats may exert similar enhancing effects on the preneoplastic response of liver sharply conflicts with the concept that phenobarbital acts as a weakly carcinogenic agent. However, the observation that pronounced tumor-promoting effects may

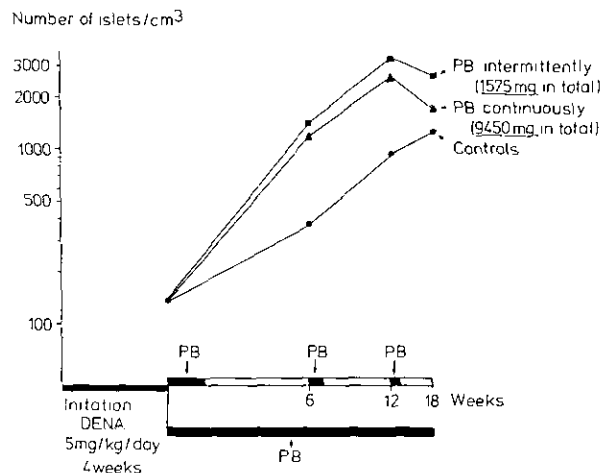


FIGURE 11. Effects of intermittent and continuous phenobarbital treatment on the number of ATPase-deficient islets in livers of rats pretreated with diethylnitrosamine. Adult female Wistar rats were pretreated with diethylnitrosamine (50 ppm in the drinking water, equivalent to an uptake of 5 mg/kg/day) for a period of 4 weeks. One group of carcinogen pretreated animals was subsequently treated continuously with phenobarbital (0.1% in the drinking water) for 18 weeks, a second group received this dose level for only 1 week at 6-week intervals. The remaining animals served as controls.

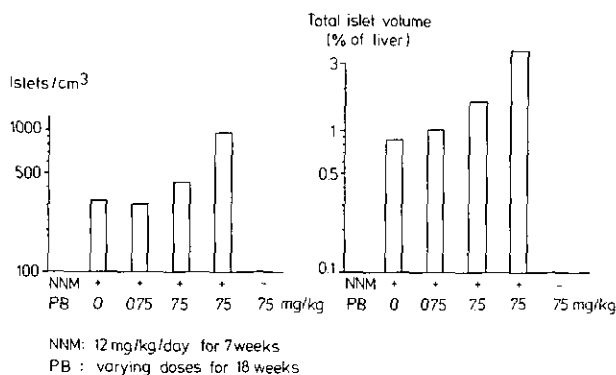


FIGURE 12. Effect of varying doses of phenobarbital on number and total volume of ATPase-deficient islets in livers of rats pretreated with *N*-nitrosomorpholine (NNM). Female Lewis rats, weighing approximately 200 g, were pretreated with NNM (120 ppm in the drinking water for a period of 7 weeks). Values indicate treatment mean (six animals/group).

also occur as a result of intermittent drug treatment could raise considerable problems in terms of the assessment of drug safety in the human situation.

Since the doses of phenobarbital usually employed in these animal studies exceed the therapeutic dose by several orders of magnitude, the assessment of the relevance of phenobarbital-mediated tumor promotion to humans would seem to necessitate an evaluation of dose-response characteristics. We have therefore investigated the dose-dependency of phenobarbital-mediated tumor promotion in rats (44). Quantitative analysis of number, total volume and size distribution of ATPase-negative and γ -glutamyltransferase-positive islets indicated that significant effects were associated with a phenobarbital dose of 75 mg/kg only (Fig. 12). Smaller exposure levels caused limited effects within control range. None of the three phenobarbital exposure levels caused any effect in livers of noninitiated animals. These latter results constitute additional evidence to indicate that the compound is devoid of carcinogenic activity. The dose-response lines, when recorded as in Figure 2, showed that in this dose region the effects approach zero. It should be noted however, that even with a threshold dose clearly defined in rodents on the basis of these studies, risk assessments may not be realistic as long as the nature of the critical cellular events and the dose levels required to produce such effects in humans remain obscure.

In the reported dose-response study with phenobarbital, induction of the microsomal mono-oxygenase system was noted in all treatment groups, followed by dose-dependent increases in liver weight. At dose levels associated with overt tumor-

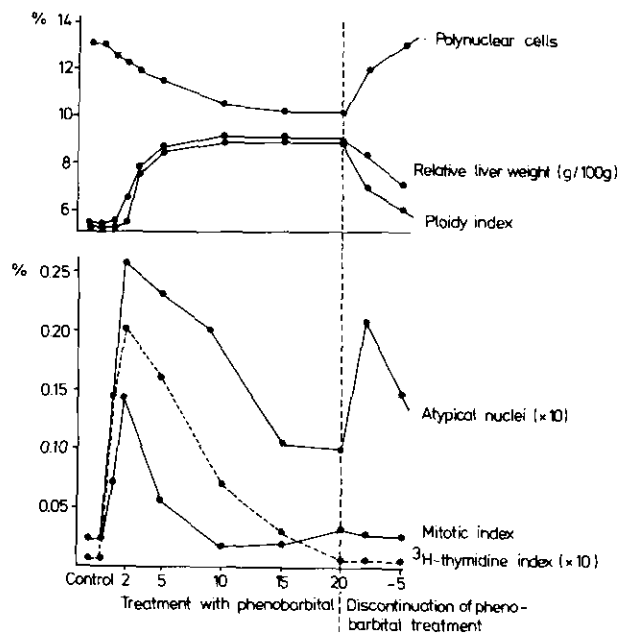


FIGURE 13. Hepatocellular changes induced by phenobarbital. Adult female NMRI mice were continuously treated with phenobarbital (0.1% in the drinking water). All cytological parameters were quantified with light microscopy on liver sections. Ploidy histograms were measured in sections and, additionally, in isolated liver nuclei. ³H-thymidine autoradiography was carried out as described by Rabes et al. (48). DNA content of nuclei was determined by microscope fluorimetry of Feulgen-stained material. Specific radioactivity in nuclei was determined by using a Leitz microscope photometer MPV (42).

promoting action, these effects were accompanied by an increased formation of hydrogen peroxide and superoxide anions in liver microsomes *in vitro* (unpublished observations). It is interesting to note that induction of reactive oxygen species has also been observed in livers of rats exposed to tumor-promoting drugs that fail to induce microsomal enzyme systems, such as clofibrate and halothane. The formation of reactive oxygen species is presently discussed as a critical phenomenon in TPA-mediated tumor promotion, since this effect appears to be related to the stimulation of cell proliferation, in particular of cellular polyploidization, and possibly also to the induction of sister-chromatid exchanges. A marked increase in hepatocellular ploidy status is also characteristic of drug-induced liver enlargement. Analysis of cytological parameters revealed that in the initial stages of liver enlargement, this phenomenon is due not only to DNA replication and endomitosis, but also to nuclear fusion in binucleated liver cells (Fig. 13). Following adaptation of the liver to the increased functional demands, and particularly after cessation of drug-treatment, there is a decline in the extent of

nuclear polyploidy associated with an increase in the number of polynucleated cells. In the course of this process atypical nuclear figures are frequently observed, which may be a reflection of aberrant nuclear divisions. It is conceivable that changes in hepatocellular ploidy status leading to abnormal chromosomal redistribution could turn heterozygous mutations homozygous. In this way, recessive oncogenic information could become phenotypically manifest (second hit). This concept provides an explanation for the observed increases in the number of islets for considerable periods of time after discontinuation of carcinogen treatment and for the impact of tumor-promoting drugs on this process. The accelerated progression of the neoplastic character of islets in livers of phenobarbital-treated animals may be similarly explained.

The induction of such critical events in rodent liver necessitates high exposure levels of tumor-promoting agents usually far in excess of the levels used in the human situation. In this context, it is interesting to note that epidemiological surveys have failed to demonstrate adverse effects of protracted phenobarbital treatment on human health (49, 50). It remains conceivable, however, that some tumor-promoting drugs could trigger critical disturbances in liver cells at dose levels approaching those used in human therapy (51).

These considerations may have important general implications for the design and interpretation of animal carcinogenicity tests. Such experiments must address the question whether tumorigenic effects are due to the action of a carcinogenic agent, or to facilitation of the expression of a pre-existing endogenous, viral or environmental oncogenic factor. Since the effects of promoters would seem to be reversible and nonexistent below threshold levels, the risks associated with these compounds should be assessed on the basis of exposure levels relevant to man.

This paper is dedicated to Professor O. Westphal on the occasion of his 70th birthday.

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