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Short Term Induction of Preneoplastic Nodules in the Rat Liver. I. The Role of 2-AAF as Selecting Agent

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Abstract—*Induction of preneoplastic nodules by a single administration of N-nitrosodiethylamine (NDEA) as initiator and selective stimulation of proliferation by 2-acetylaminofluorene (2-AAF) in combination with partial hepatectomy was used to study the effect of different doses of NDEA and 2-AAF on number and size of the nodules. There was a positive relationship between the number of preneoplastic nodules and the dose of NDEA. The marked increased capacity to proliferate with increasing amounts of NDEA suggests an acquired genetically fixed phenomenon. Morphologically there was a striking uniformity of the nodules induced by different doses of NDEA during the first 2 weeks of development, and stimulated by different doses of 2-AAF. This suggests that possible subpopulations were not formed at all during the experimental period of 2 weeks. Alternatively, a genetic heterogenicity may be expressed at a later stage or under different conditions.*

The effect of 2-AAF reached a plateau at 5 mg/kg body weight; higher concentrations did not influence the number and size of the nodules. Although there was a steady increase in size of the nodules during early development, the number of nodules reached a maximum value at 2 days after partial hepatectomy. It is concluded that 2-AAF only permits selective outgrowth of previously initiated cells by its cytotoxic effects on the surrounding normal tissue and that the carcinogenic properties of 2-AAF in this model do not come to expression.

INTRODUCTION

THE DEVELOPMENT of cancer is a multistep process in which initiation of genotypically altered cells by a carcinogenic agent is the primary event, which is most likely based on a persistent, perhaps irreversible alteration of DNA [1–3]. The initiated cells may remain latent for a long period of time and there is considerable evidence that progression to an autonomous cancer occurs by a sequential series of organizational, structural and biochemical changes of the cells involved [4].

Experimental induction of neoplastic changes with chemicals in the rodent liver provides a good model to study initiation and the effects of various modifying factors such as promotion on the different stages of liver neoplasms. There is ample evidence to support the hypothesis that chemically induced liver cell carcinomas in the rodents are preceded by and most likely arise from early occurring focal areas of altered liver cells [5–8]. The various characteristics of these presumptive preneoplastic and premalignant lesions exhibit a wide variation which depends mainly on age of the lesions and the experimental regimen [6, 7, 9, 10]. The lesions are commonly described as areas of cellular alterations, hyperplastic foci, enzyme deficient islands or (pre)neoplastic (hyperplastic) nodules [8, 11, 12].

Craddock [13] and Scherer and Emmelot [14] have demonstrated that a single injection of an alkylating liver carcinogen provides a good model for the initiation of carcinogenesis in the liver. Solt and Farber [15] showed a rapid formation of hyperplastic liver nodules in rats after a single dose of NDEA followed by partial hepatectomy and selective stimulation of proliferation of the preneoplastic hepatocytes by a short period of feeding a diet containing a small amount of 2-AAF. The advantage of this experimental procedure is the short period necessary for the development of the preneoplastic nodules and the obtained distinct growth synchrony of the lesions which permits accurate study of the successive stages [8, 16].

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Tsuda and Farber [17] have shown that a variety of carcinogens of different types can induce early preneoplastic lesions after a single dose in combination with a stimulus for liver cell proliferation, whereas non-carcinogens were negative; this indicates that induction of preneoplastic liver changes is potentially a new *in vivo* short term assay for genotoxic carcinogens in which the end point has direct relevance to cancer.

As there is insufficient quantitative data on the early stages of development and the role of selective stimulation of proliferation, in this paper an analysis is presented on the relationship between different doses of NDEA as initiating agent and the number and size of the liver nodules. In addition quantitative features of early stages and the effect of varying doses of 2-AAF as selective stimulator of liver cell proliferation were studied.

MATERIALS AND METHODS

Animals

Male, SPF-derived Wistar/RIV rats (180–220 g) were housed in stainless steel insulators in groups of 3–5 in macrolon cages. Food pellets containing not less than 22% protein (Muracon, Trouw and Co., Putten, The Netherlands) and tapwater were available *ad libitum*. Analysis of the diets for various *N*-nitroso-compounds and aflatoxins was negative except for one charge that contained nitrosodimethylamine at a concentration below 1.0 μg/kg.

Experimental design (*Fig.* 1)

Experiment 1. In order to evaluate the dose–response relationship of NDEA on induction of preneoplastic nodules 30 rats were divided into 3 groups of 5 and 1 group of 15 animals, which received respectively 0, 10, 50 and 200 mg NDEA/kg body weight (b.w.) (dissolved in 0.9% saline) as a single dose i.p. and a daily dose by gavage of 20 mg 2-AAF/kg body weight (b.w.) suspended in carboxymethyl cellulose 1% (CMC) from day 14 onwards. Twenty-one days after NDEA administration 3/4 hepatectomy was performed under light ether anesthesia according to the method of Higgins and Anderson [18]. At day 33 the animals were anesthetized by i.v. injected pentobarbital-sodium and subjected to the autopsy procedure.

Experiment 2. To study the early development of preneoplastic nodules longitudinally in time, 27 rats were divided into 9 groups of 3 animals, which received a single dose of 200 mg NDEA/kg b.w. i.p. From day 14 onwards a daily dose of 20 mg 2-AAF/kg b.w. was given by gavage. Hepatectomy was performed at day 21. At day 21, 22, 23, 24, 25, 26, 27, 31 and 33 groups of 3 rats were subjected to autopsy.

Experiment 3. To study the effect of varying doses of 2-AAF on the development of the preneoplastic nodules, 30 rats received each 200 mg NDEA/kg b.w. i.p. Subsequently the rats were divided into 6 groups of 5 animals which from the end of week 2 onwards received a daily dose of 0, 2.5, 5, 10, 15 or 20 mg 2-AAF/kg b.w. Hepatectomy was per-

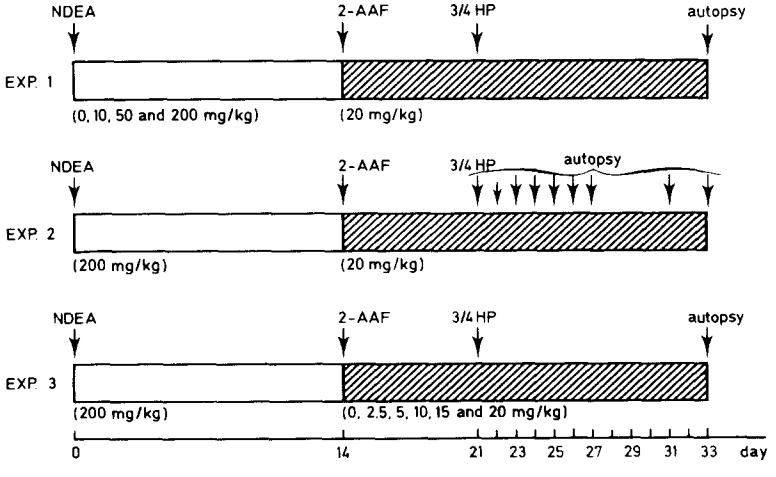

Fig. 1. *Scheme of experimental regimen as described in Materials and Methods. NDEA: single administration of* N*-nitrosodiethylamine i.p. at the indicated dose levels: hatched bars: daily administration by gavage of* 2*-AAF* (2*-acetylaminofluorene*) *at the indicated dose levels;* 3/4 *HP: partial hepatectomy.*

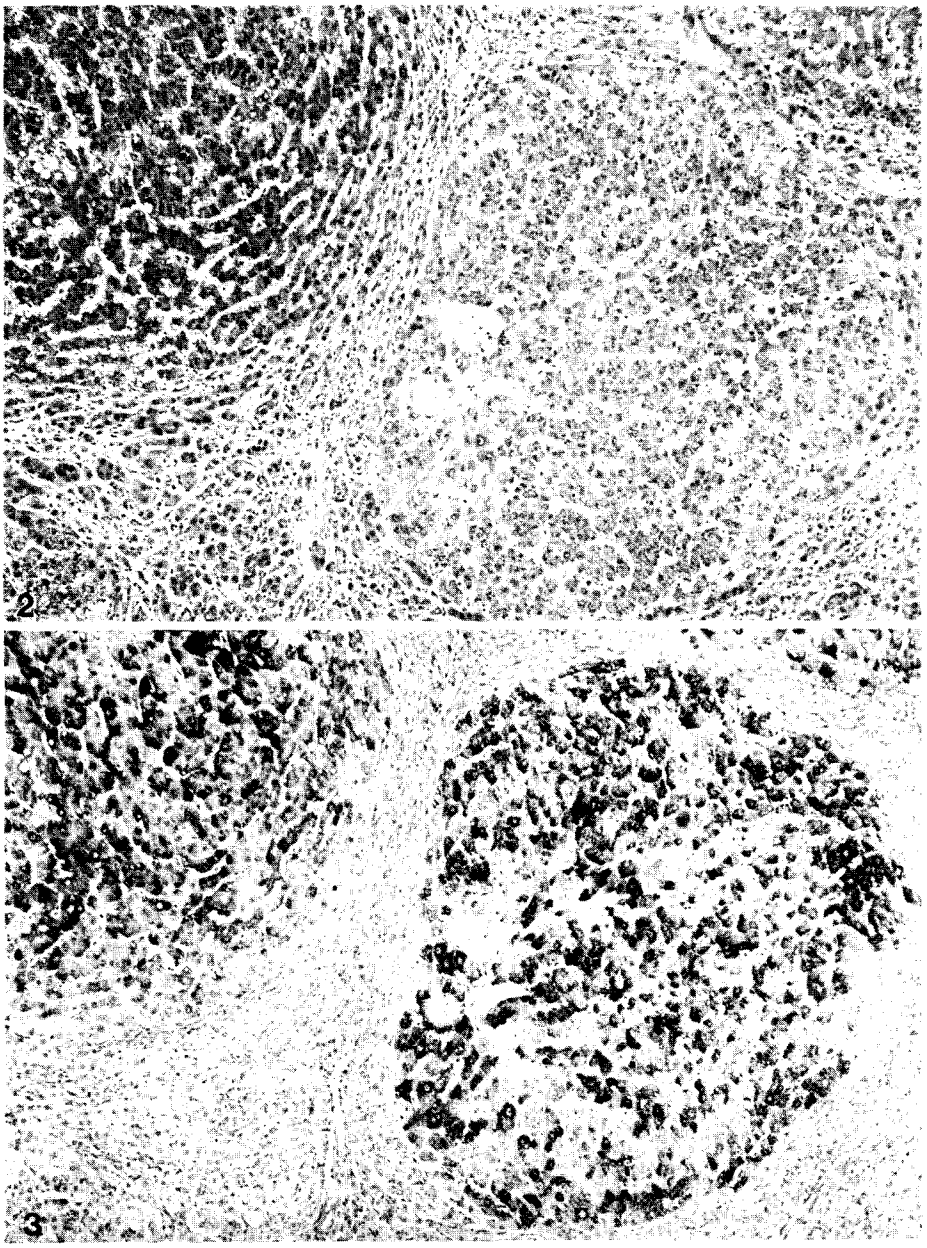

Fig. 2. *Liver of a rat at* day 33 *treated with* 200 mg NDEA/kg b.w. *and* 20 mg 2-*AAF*/kg b.w. *The picture shows preneoplastic nodules compressing the surrounding non-nodular liver tissue. H.E.* ×80.

Fig. 3. Idem *Fig.* 2. *In this serial section the nodular cells exhibit an abundancy of glycogen as shown by PAS staining, whereas the surrounding tissue is negative. P.A.S.* ×80.

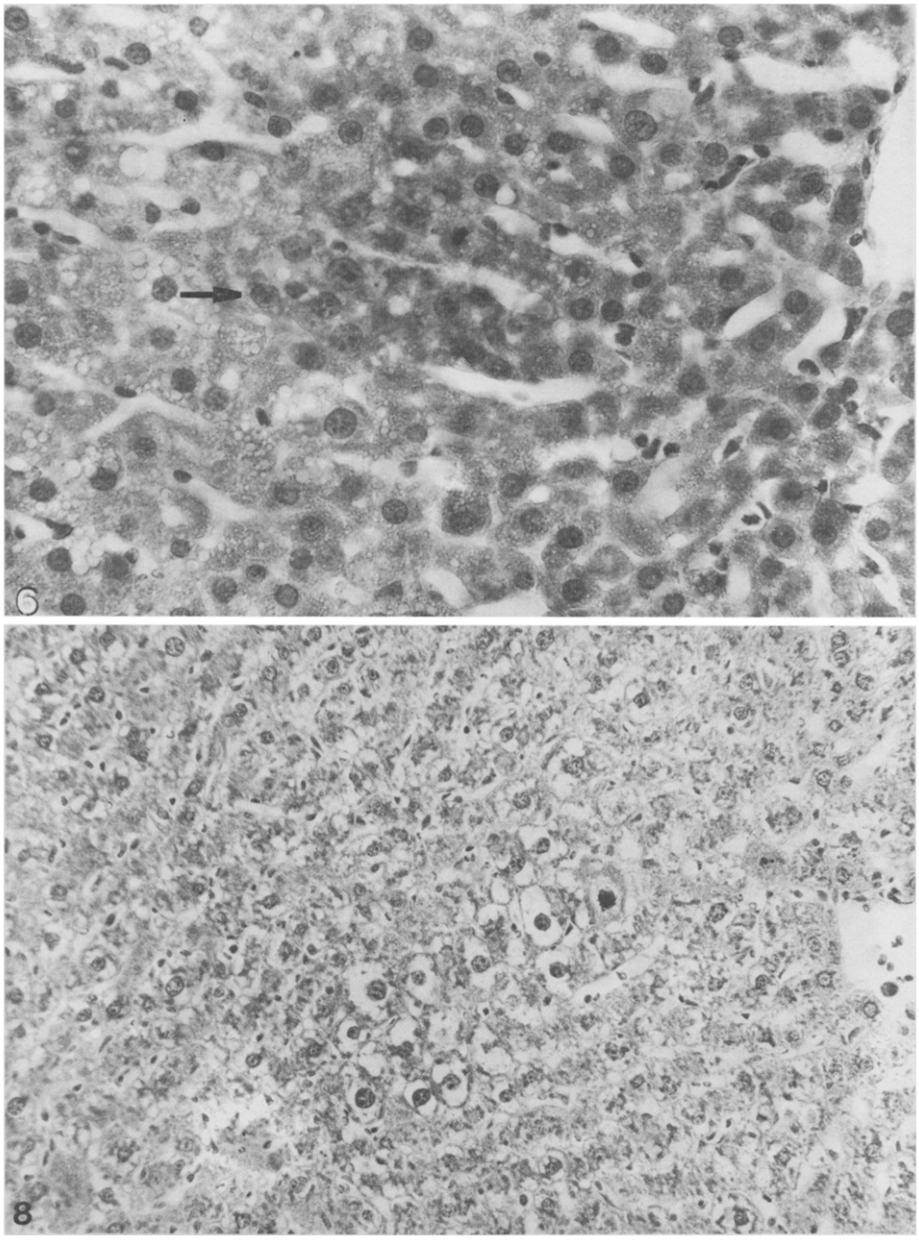

Fig. 6. *A section from the liver of a rat on* day 25 *treated with* 200 mg *NDEA*/kg b.w. *and* 20 mg 2-*AAF*/kg b.w. *In the centre of the picture a small group of cells shows a characteristic basophilic cytoplasm and enlarged nucleoli* (*arrow*). *H.E.* × 320.

Fig. 8. *Liver of a rat* day 33 *treated with* 200 mg *NDEA*/kg b.w. *and* 2.5 mg 2-*AAF*/kg b.w. *showing a small group of altered cells having a clear, vesicular cytoplasm and enlarged nuclei. H.E.* × 200.

formed at day 21. At day 33 the rats were subjected to autopsy. Only data from animals that survived the full experimental term were included in the calculations.

Autopsy procedure

Prior to autopsy the animals were fasted overnight and anesthetized by i.v. injected pentobarbital-sodium. Thereafter the liver was perfused via the portal vein with phosphate buffered fixative (4% formaldehyde and 1% glutaraldehyde). Before perfusing the liver, the right caudal lobe was ligated, removed and frozen in liquid nitrogen to assess histochemically the glycogen content by the PAS/PAS–diastase method and the activity of some marker enzymes for nodules such as glucose-6-phosphatase, canalicular adenosinetriphosphatase and γ-glutamyltranspeptidase.

Quantification of the preneoplastic nodules

For quantification of the preneoplastic nodules, defined as focal areas of proliferating hepatocytes that have acquired a resistance to certain cytotoxic compounds, representative samples were taken from each liver lobe. Paraplast sections (5 μm) were stained with haematoxylin and eosin (HE). The number of nodules per cm^3 liver tissue and their volume was established according to the method described by Scherer *et al.* [19].

RESULTS

Experiment 1

Gross examination of rats that received 200 mg NDEA/kg b.w. revealed at day 33 multiple pale-yellowish elevated foci on the liver surface, ranging in diameter from very small up to 3 mm. On cut surface the foci were evenly distributed throughout the various liver lobes. Microscopically the nodules were easy to detect by increased cytoplasmic basophilia and they were randomly scattered within the parenchyma of the liver lobes. There was marked compression of the surrounding tissue (Fig. 2). The architecture of the nodules was slightly irregular: the liver cells were generally arranged in plates of two or three layers which formed frequently small tubular or cystic structures. The sinuses within the nodules, though locally dilated, looked regular and were lined by normally appearing endothelial and Kupffer cells. The altered liver cells which were fairly uniform in appearance displayed an intense basophilic, mostly grannular, cytoplasm and had prominent and sharply outlined cell borders. Occasionally a small group of cells within the nodule exhibited a different smooth basophilic cytoplasm whereas scattered throughout the nodule some individual cells showed intracytoplasmic lipid droplets. The size of the nuclei varied somewhat and because the chromatin was clumped to the nuclear membrane, they seemed rather empty and vesicular. The most notable feature of the nuclei was the presence of a large prominent nucleolus. In addition, many, sometimes atypical, mitotic figures were seen. The altered liver cells often contained an abundancy of glycogen in cytoplasm as demonstrated by the PAS stain (Fig. 3). Histochemically, the nodules were uniformly characterized by the presence of γ-glutamyltranspeptidase and the absence of adenosinetriphosphatase and glucose-6-phosphatase. The gross and microscopic morphology of the liver nodules in rats that received 10 and 50 mg NDEA/kg b.w. did not differ from the nodules of the 200 mg/kg b.w. group except for their size. Also the location of the small nodules was not restricted to a particular area of the liver lobule.

The effect of varying doses of NDEA on the number and volume of the preneoplastic nodules is shown in Fig. 4.

A distinct, positive relationship exists between the amount of NDEA administered and the volume and the number of nodules per cm^3 tissue. There was a remarkably uniform response to treatment of the individual rats of the various groups.

In the livers of rats that received 10 mg NDEA/kg b.w. the nodules were small, occupying only a small fraction of the section of the liver, with slight or no compression of the

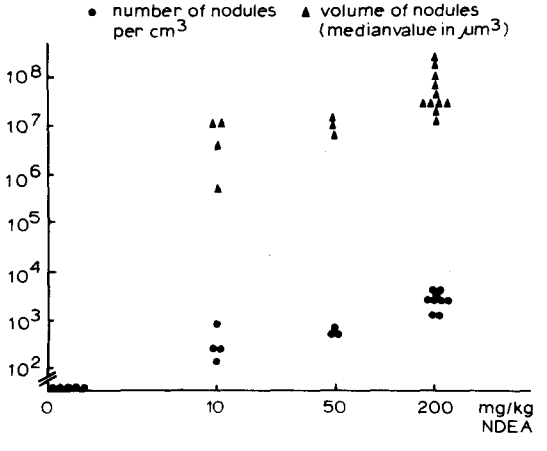

Fig. 4. Number and volume of preneoplastic liver nodules on day 33, *initiated by different doses of NDEA, and selectively stimulated by* 20 mg 2-*AAF*/kg b.w. *and partial hepatectomy.*

adjacent parenchyma; whereas in the animals that received 200 mg NDEA/kg b.w. the nodules were large, with only a very small rim of compressed residual non-nodular liver cells.

In rats that were subjected to partial hepatectomy and 2-AAF treatment without prior NDEA administration the microscopy of the liver revealed occasionally a rather large nodule.

Experiment 2

The development of the nodules during the first 12 days after hepatectomy is illustrated in Fig. 5. In the livers studied one day after partial hepatectomy the picture was not clear and only occasionally a few preneoplastic cells could tentatively be identified. From 2 days after hepatectomy, however, many small groups of basophilic hepatocytes with characteristically large nucleoli could be recognized (Fig. 6). The individual cells were similar to the preneoplastic cells observed at day 33 after NDEA treatment as described before. During the subsequent development the altered cells, of which the morphology did not differ from previous stages, proliferated and the surrounding liver tissue became gradually compressed. Thus, the early development of the nodules is mainly characterized by growth. The number of nodules per cm^3, however, appeared to decrease from 2 days after hepatectomy (10^4) up to day 33 (2×10^3). However, compared with the about three-fold increase in liver weight during the same period from approximately 2 up to 6 g the absolute number of nodules per liver remained remarkably constant.

Experiment 3

The effect of various doses of 2-AAF on the number and size of nodules which were initially induced by a single dose of 200 mg NDEA body weight is illustrated in Fig. 7. These results showed clearly that the effect of increasing dose levels of 2-AAF as selective stimulator of proliferation soon reaches a plateau. Without administration of 2-AAF no nodules were present. A daily dose of 2.5 mg 2-AAF/kg b.w. stimulates growth of only a small number of nodules. The number of nodules per cm^3 reached its maximum at 5 mg 2-AAF/kg b.w. and remained constant at dose levels of 10, 15 and 20 mg 2-AAF/kg b.w.

The size of the nodules on the other hand depended more on the amount of 2-AAF administered; there was a dose-related increase in size with a maximum at the level of 15 mg 2-AAF/kg b.w. The nodules in the liver from animals that received a daily dose of 20 mg 2-AAF/kg b.w. remained smaller. The general condition of the rats treated daily with 20 mg 2-AAF kg b.w. markedly deteriorated immediately after the partial hepatec-

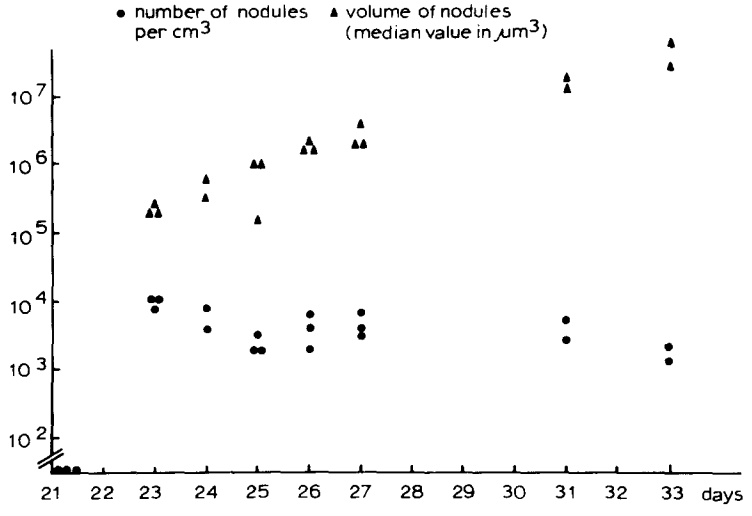

Fig. 5. Number and volume of preneoplastic liver nodules, initiated by 200 mg *NDEA*/kg b.w. *and selectively stimulated by* 20 mg *2-AAF*/kg b.w. *at various days after partial hepatectomy on* day 21.

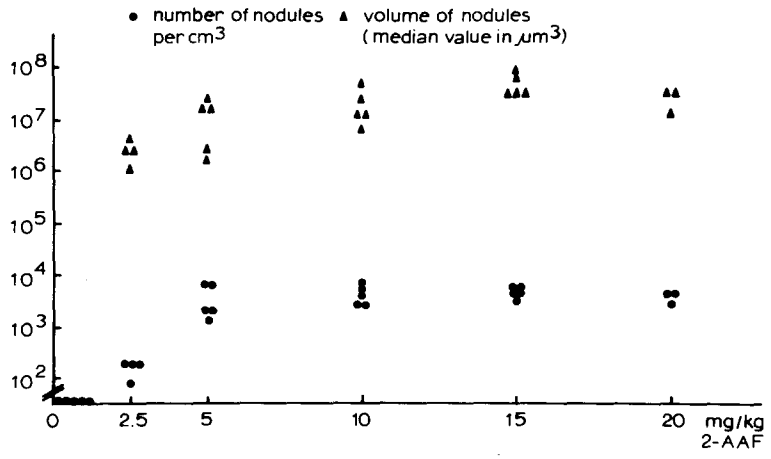

Fig. 7. Number and volume of preneoplastic liver nodules, initiated by 200 mg *NDEA*/kg b.w. *and selectively stimulated by various doses of 2-AAF, and partial hepatectomy.*

tomy and a few animals died from the toxic action of 2-AAF. The microscopic morphology of the nodules in animals treated with 2.5 mg 2-AAF/kg b.w. differed clearly from the nodules of animals treated with 5 mg 2-AAF/kg b.w. or more. At the level of 2.5 mg 2-AAF/kg b.w. the liver changes consisted at day 33 of unconspicuous, small islands of altered cells having a clear or foamy or sometimes vesicular cytoplasm, exhibiting no distinct basophilia. The nucleus only occasionally contained an enlarged, prominent nucleolus (Fig. 8). The microscopic appearance of the nodules in animals treated with 5, 10 and 15 and 20 mg 2-AAF/kg b.w. was identical with that of the nodules described earlier in experiment 1. The liver cells in the surrounding liver tissue were small and did generally not contain any glycogen after fasting. During the 2-AAF treatment the normal liver cells exhibited progressive signs of degeneration. There was a diffuse proliferation of oval cells which increased in severity with increasing levels of 2-AAF; this proliferation started mainly after partial hepatectomy and accompanied the proliferation of the altered liver cells.

The liver taken at hepatectomy from animals given NDEA without 2-AAF treatment did not show any microscopic lesions which indicated that the toxic effect of the initial NDEA treatment had disappeared.

DISCUSSION

The results of this study confirm that preneoplastic liver nodules arise within a short time from liver cells that are initially altered by a single dose of the potent liver carcinogen NDEA, followed by an experimental regimen consisting of partial hepatectomy that provides a powerful stimulus of liver cell proliferation and exposure to low levels of 2-AAF that is toxic to normal hepatocytes and inhibits their proliferation. Proliferation of bile duct cells was apparently not inhibited by 2-AAF. This reactive phenomenon is most likely caused by the inability of the oval cells to metabolize 2-AAF into a cytotoxic derivative.

Some of these preneoplastic nodules progress gradually into autonomously growing liver cell tumours as shown by Solt *et al.* [8]. In our laboratory 3 out of 7 animals, that received 200 mg NDEA/kg as a single dose, developed liver cell carcinomas within 1 yr when kept under normal conditions after day 33 (unpublished data).

The increase of the number of nodules with increasing doses of NDEA is in agreement with the results of Solt and Farber [15]. In contrast with these results Scherer and Emmelot [7] observed the presence of a plateau from a dose level of 50 mg NDEA/kg b.w. In their experiments, however, they performed partial hepatectomy 20–24 hr prior to the NDEA administration, so only one-third of the original liver mass was available. Moreover, the NDEA was given at a time when the majority of cells were in the prereplicating phase, thus very sensitive to carcinogens [20]. The increase of the volume of the nodules proportionally with the amount of NDEA administered initially cannot be explained and may point to a genetically fixed capacity of the preneoplastic cells to pro-

liferate faster, or to an increased resistence of these cells to the toxic action of 2-AAF as the doses of the initiating carcinogen are higher. An increase in size of the nodules with increasing dose levels was also observed by Scherer and Emmelot [7] and Rabes and Szymkowiak [21] and these authors explained this increase in size by the dose related increased toxicity of the carcinogen to the normal liver cells. In our study, however, the carcinogen was administered 3 weeks prior to hepatectomy and to the subsequent outgrowth of the altered liver cells. At the moment of hepatectomy the toxic effects of NDEA were no longer detectable microscopically. Nevertheless the nodules induced by higher doses of NDEA proliferated faster although the amount of 2-AAF and thus the toxic load on the liver for the selection pressure was similar at the various dose levels of NDEA.

The remarkable uniformity of the preneoplastic nodules suggests that the nodules consist of clones of cells [22] initially altered by NDEA on the same DNA target. This absence of differentiation of nodules in our experiments suggests that stepwise progression to a more heterogeneous population of nodules and ultimately liver cell carcinoma does not take place during the first weeks of development. However, despite this phenotypic uniformity during early development only a few nodules finally progress to a liver cell carcinoma. For this progression to liver cell carcinoma there are two extreme possibilities: only a few cells have the capacity to progress (genetic heterogenicity) or many nodules have the capacity to progress (at risk) but only a few receive stochastically the event(s) related to further progression. The genetic heterogenicity between the nodules may be expressed at a later stage or under different experimental conditions. This genetic variation may for instance express itself in differences in proliferation activity, enzyme patterns or ultrastructure [8, 21, 23, 24]. Alternatively, reported phenotypical differences [25] may be masked in this study by cellular features primarily associated with rapid growth; e.g., basophilia and an enlarged nucleolus. The hypothesis that a single dose of a carcinogen induces an irreversible genetically fixed effect in the DNA [26] is further evidenced by experiments in which NDEA was given up to 4 months prior to the procedure of selective proliferation, e.g., partial hepatectomy and 2-AAF treatment. From these experiments it is concluded that altered liver cells remain dormant for a long period of time and the final number and size of the nodules is apparently not influenced by the length of the previous dormant state (van der Heijden, unpublished data).

Solt *et al.* [8] and Ogawa *et al.* [16] reported that no nodules could be observed when no NDEA was given. However, in a previous study Solt and Farber [15] reported that a few basophilic foci were rarely seen without pretreatment with NDEA, which is in agreement with our results. Because 2-AAF possesses carcinogenic as well as toxic properties, 2-AAF administration especially in combination with hepatectomy when the cells are particularly vulnerable to the genotoxic action of a carcinogen may result in the induction of altered cells [19, 20] which consequently can develop into preneoplastic nodules that are indistinguishable from NDEA initiated nodules. However, the number of nodules was unaffected by the dose rate of 2-AAF (5–20 mg/kg b.w.) or duration of 2-AAF treatment. This indicates that 2-AAF in this model did not act as a syncarcinogen as it did not contribute in nodule induction as compared with 200 mg NDEA/kg b.w. Moreover, the results suggest that all initiated cells were stimulated by the experimental regimen to grow out into nodules.

The sharp increase of the number of nodules from 2.5 to 5 mg/kg b.w. indicates that there is a threshold for the 2-AAF dependent suppression of proliferation of normal hepatocytes leading to the stimulation and expression of only a limited number of initiated cells. These foci were also smaller and showed a different and less basophilic cytoplasm, which indicates slower proliferation.

The mechanism by which 2-AAF inhibits proliferation of normal liver cells while permitting growth of only preneoplastic nodules remains obscure. Farber *et al.* [27] found that altered cells were resistent to the acute necrogenic effect of the hepatotoxins CCl_4, *N*-nitrosodimethylamine and 2-AAF. They observed a decreased uptake of ^{14}C-labelled 2-AAF by nodular cells with a subsequent decrease in binding to cellular macromolecules, either as result of the reduced uptake or altered metabolism of the compound. Tatematsu *et al.* [28] tested a series of carcinogens and non-carcinogens as possible candidates to replace 2-AAF and they concluded from their results that only carcinogens have the potency to act as selective stimulator. This is in agreement with preliminary results in our laboratory which show that high doses of the non-carcinogenic hepatotoxin paracetamol

(up to 1000 mg/kg b.w.) failed to permit proliferation of normal cells as well as preneoplastic cells. Our data suggest that high doses of 2-AAF (20 mg/kg b.w.) results in smaller nodules (Fig. 4) which indicates that in nodular cells apparently toxic 2-AAF metabolites are also formed in sufficiently high concentration to inhibit proliferation. The available data thus indicate that the resistance of altered cells to 2-AAF depends on the inability of the altered cells to produce particular metabolites toxic to specialised cellular functions (e.g., mitosis).

In conclusion, this model is very promising as a short-term *in vivo* test for detection of carcinogens and to study complex interactions of toxic and carcinogenic substance on hepatocarcinogenesis. The mechanisms by which the various parts of this model act, such as 'initiation' and 'promotion', are still not clear. This study may attribute to further understanding.

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