DNA Synthesis, Apoptosis, and Phenotypic Expression as Determinants of Growth of Altered Foci in Rat Liver during Phenobarbital Promotion

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ABSTRACT

Carcinogenesis was initiated in female rat liver by a single dose of N-nitrosomorpholine; subsequently phenobarbital (PB) was administered via the diet at a daily dose of 50 mg/kg body weight for up to 49 weeks. Subgroups of rats were left untreated after 10 or 28 weeks on PB, produced the following changes: (a) accelerated appearance of neoplastic nodules and hepatocellular carcinoma (from 28 weeks onwards); (b) phenotypic changes in altered foci such as a shift from clear to eosinophilic appearance, enhanced expression of γ-glutamyltranspeptidase and other markers, and more distinct borders from surrounding liver; (c) an increase in foci number; and (d) accelerated foci enlargement. The increase in foci number was found to be due to increased phenotypic expression of foci.

DNA synthesis was measured by [3H]thymidine labeling at multiple time points. The rate of DNA synthesis was always approximately 10-fold higher in foci than in surrounding liver tissue. Despite this, after N-nitrosomorpholine alone foci grew little before 18-24 weeks. Continuous treatment with PB did not produce a persistent further increase of DNA synthesis in foci, although it accelerated foci growth. Furthermore, at early stages small and larger foci showed similar DNA synthesis activity. These findings indicate that the rate of cell replication as measured by DNA synthesis is not the only determinant of the growth rate of foci. Further studies showed that foci with indistinct borders (reflecting weak expression of the altered phenotype) grew much less than foci with distinct borders; this was at least in part due to an increased rate of cell death by apoptosis found in foci with indistinct borders. In conclusion, besides cell replication, apoptosis and the extent of phenotypic expression (remodeling) determine the growth rate of foci. Foci with weak phenotypic expression predominated after N-nitrosomorpholine alone; in these, a high incidence of apoptosis counterbalanced cell replication. In contrast, during PB treatment foci with strong phenotypic expression predominated; in these, apoptotic activity was lower and the high replicative activity could manifest itself.

Finally, all effects of PB on foci were largely although not completely reversible upon cessation of treatment; as a result phenotypic expression declined, and "remodeling" foci with high apoptotic activity predominated again.

INTRODUCTION

Tumor promotion has been recognized as an important event in the multistage process of carcinogenesis (1-8). It can be affected by many nongenotoxic carcinogens. Studies on the mechanisms of tumor promotion by chemical agents are important in order to understand the complex process of carcinogenesis, to assess the health risks of exposure to nongenotoxic carcinogens, and to develop simple testing procedures for discovery of this class of agents. The liver offers a suitable system for mechanistic studies because in this organ the putative progeny of initiated cells, the target for tumor promotion, can be readily identified as foci of phenotypically altered cells. Their biology has been elucidated to some extent (2, 5-8). Furthermore, the pioneering studies of Peraino et al. (1) have provided a suitable model tumor promoter, i.e., PB, a compound with little if any hepatotoxic activity.

Altered foci show accelerated enlargement in response to PB. How this enlargement is effected is not clear. We and others have described previously that altered hepatocytes in foci exhibit such higher proliferative activity than normal hepatocytes (9-16). Single doses of PB and other nongenotoxic carcinogens (tumor promoters) caused further increases of cell proliferation in foci (9), but little is known on proliferation during long term PB treatment. Therefore, we studied the time course of foci enlargement and tried to correlate it with proliferative activity in foci at multiple time points covering the entire period from initiation to the appearance of tumors in PB-treated rats. Since the results suggested that the extent of cell replication in foci is not the only determinant of foci growth, we have also investigated the role of cell death through apoptosis and of phenotypic "remodeling" for the enlargement of foci.

A variety of models of liver tumor promotion are currently under study and differ in important biological characteristics (8). If these are not taken into account, confusion may arise when comparing the observations from different models. Therefore, we investigated and describe here some relevant biological features of our model to provide a sound basis for interpretation of the cell kinetic data.

MATERIALS AND METHODS

Animals. Female specific pathogen-free Wistar rats, 3-4 weeks old, were obtained from Zentralinstitut für Versuchstierzucht (Hannover, Germany). They were housed in Makrolon cages, 3/cage, in a climatized room. If not stated otherwise, the light-dark rhythm was reversed, with lights off from 9 a.m. to 9 p.m. Food (Altromin 1320; Lage, Germany) was provided ad libitum, except when hepatic DNA synthesis was to be synchronized before administration of NNM or [3H]thymidine. For this purpose rats were adapted to a feeding rhythm for at least 2 weeks, as described before (17). They received food from 9 a.m. to 9 p.m. before NNM and from 9 a.m. to 5 p.m. before [3H]thymidine. Water was always available. Rats were killed between 8 a.m. and 10 a.m. by decapitation. The livers were quickly excised and weighed, and specimens were stored at -15°C for determination of DNA; other specimens were fixed in ice-cold acetone for histochemistry and immunocytochemistry and in formalin or Carnoy's solution for histological analysis.

Treatment and Experimental Design. The experimental design, with number of rats/group and sacrifice times, is shown in Fig. 1. NNM (250 mg/kg) (Serva Biochemica, Heidelberg) was administered p.o. as an aqueous solution when the rats were about 6 weeks old (body weight, 80-100 g). It was applied at 8. p.m. Following a recovery period of 8 weeks, rats received either basal diet or a diet containing PB. Food consumption was measured twice weekly or (after the initial 2 months) once weekly, and the concentration of PB in the diet was adjusted to provide a daily dose of 50 mg/kg body weight. In additional groups PB diet was replaced by basal diet at either 10 or 28 weeks (Fig. 1). Further animals were left completely untreated or received PB diet for control.

[3H]Thymidine (6.7 Ci/mmol; NEN Frankfurt, Germany) was in-

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2 The abbreviations used are: PB, phenobarbital; AB, apoptotic body; DB, distinct border; GGT, γ-glutamyltranspeptidase; INDB, indistinct border; NNM, N-nitrosomorpholine; H&E, hematoxylin and eosin; LI, labeling index; PAS, periodic acid-Schiff reaction.
reflected the number of cells replicating/day. This assumption was rate. The following assumptions were made, (a) The LI obtained focus after experimental period / (in days), and K = daily proliferation of cells/focus at start (8 weeks after NNM, / = 0), N = number of cells/ at the various times of investigation to calculate their (possible) growthrate. The following formula was used: N = N0−e−*, with N0 = number H&E-stained sections as described previously (23), and their incidence/ were recorded as a focus when no compression of surrounding tissue were increased in PB-treated animals at all time points studied (Fig. 2b); this increase was on average 20% in the first weeks of treatment and became larger (approximately 35%) at the end of the experimental period; the late increase may in part result from amplification of focal tissue and formation of tumors (see below). (The decrease of liver weights between 6 and 10 weeks is due to a change in the feeding regimen, see “Materials and Methods” and legend to Fig. 2.) Liver DNA contents also considered justified because daily hepatic DNA synthesis was sychronized (see above). (b) The animals were killed 12–24 h after [3H]thymidine, at a time well beyond the maximum of mitotic activity at 3 a.m. (17); therefore, it was assumed that all labeled hepatocytes had undergone mitosis. This has been verified previously (24). (c) Furthermore, we assumed exponential growth of foci, with identical probabilities to proliferate of all altered cells in a focus, and no occurrence of cell loss. N0 was obtained from the average size of foci; number of focus cells/cross-section was transformed into number of cells/focus volume, assuming spherical shapes. Calculations were made separately for all intervals between two adjacent sacrifice times, and for estimation of K the means of LIs obtained at these two time points were used. Finally, results were transformed into number of cells/cross-section in order to facilitate comparison with foci sizes found experimentally. Further details are published elsewhere (25).

Statistics. Means and SD are given. The significance of differences was checked with the t test (Student). Apoptotic counts are indicated with 95% confidence limits.

RESULTS

Body and Liver Growth. After NNM treatment body weights increased less than in control animals (not shown). PB somewhat accelerated body growth (Fig. 2a). Relative liver weights were increased in PB-treated animals at all time points studied (Fig. 2b); this increase was on average 20% in the first weeks of treatment and became larger (approximately 35%) at the end of the experimental period; the late increase may in part result from amplification of focal tissue and formation of tumors (see below). (The decrease of liver weights between 6 and 10 weeks is due to a change in the feeding regimen, see “Materials and Methods” and legend to Fig. 2.) Liver DNA contents also
increased during PB application (Fig. 2c); the increase was small (approximately 10%) and not statistically significant during initial weeks but was more pronounced later. When PB treatment was stopped after 10 or 28 weeks, liver size returned to control levels within 2 weeks; likewise, the increased DNA contents regressed but this seemed to occur more slowly (Fig. 2, b and c).

Morphology and Enzyme Markers of Foci. H&E-stained sections from NNMTreated rat liver contained foci of altered cells at all times investigated. Changes with time and PB treatment are summarized in Table 1. In rats treated with NNM alone, clear cell foci predominated (Fig. 3a); later (36 weeks or more after NNM) eosinophilic foci and a few small "tigroid" foci appeared (Fig. 3b). Following PB treatment, foci cells tended to be enlarged and their cytoplasm lost its clearness and became of ground-glass appearance (Fig. 3c), although excessive glycogen storage could still be demonstrated in serial sections by the PAS method (see below). After 10 weeks and more of PB, most foci were composed of acidophilic and diffusely basophilic hepatocytes; intermediary phenotypes were also found. From 18 weeks onward, basophilic foci, usually with small cells, appeared. This sequential appearance of various focus phenotypes resembles that described after repeated doses of genotoxic carcinogens (26). Virtually no tigroid foci were observed in rats treated with NNMTreatment alone approximately 50% of foci detectable in each pair of sections were GGT positive. Two weeks of PB treatment did not result in major increases in the total number of foci, but approximately 90% of foci now expressed GGT.

Table 1 Occurrence of various foci types in H&E-stained sections after treatment with PB

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Clear</th>
<th>Clear-acidophilic</th>
<th>Acidophilic</th>
<th>Acidophilic-basophilic</th>
<th>Basophilic (diffuse)</th>
<th>Basophilic (tigroid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNM → 0 (8–12 wk)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(++)</td>
<td>+</td>
<td>(++)</td>
</tr>
<tr>
<td>NNM → 0 (18–42 wk)</td>
<td>++</td>
<td>+</td>
<td>(++)</td>
<td>(++)</td>
<td>+</td>
<td>(++)</td>
</tr>
<tr>
<td>NNM → 0 (57 wk)</td>
<td>++</td>
<td>+</td>
<td>(++)</td>
<td>(++)</td>
<td>+</td>
<td>(++)</td>
</tr>
<tr>
<td>NNM → PB (2 wk)</td>
<td></td>
<td>++</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NNM → PB (10–34 wk)</td>
<td>(+)</td>
<td>+</td>
<td>++</td>
<td>(++)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>NNM → PB (49 wk)</td>
<td>(+)</td>
<td>+</td>
<td>++</td>
<td>(++)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>NNM → PB (10 wk) → 0 (2–6 wk)</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>(++)</td>
<td>+</td>
<td>(++)</td>
</tr>
</tbody>
</table>

* Mixed populations with both cell types including "intermediate" cell types.
++ predominant; +, less frequent; (+), rare.

Growth Kinetics of Foci. GGT-positive and -negative foci (or negative parts of foci, see above) were counted and sized. Histograms are shown in Fig. 5, and number and average size of foci are in Fig. 6. After NNMTreatment alone foci number/cm² tissue section remained essentially constant throughout the entire period of observation, and no evidence of foci enlargement was noted during the initial 10–16 weeks. Thereafter, i.e., 18–24 weeks after initiation, some foci exhibited considerable steady growth, although some small foci were always present (Fig. 5). As a result average focus size increased (Fig. 6).

PB treatment caused a rapid increase in the number of foci within 4 weeks, and a more gradual increase thereafter, resulting in 5 times more foci detectable/cm² section area than without PB treatment. Average size of foci showed no change during the initial 6 weeks of PB feeding but then gradually increased up to 10-fold at 49 weeks (Fig. 6). It is of interest to note that even at 49 weeks of PB treatment small foci were found (Fig. 5). Apparently, growth rates of individual foci varied dramatically. As a result of increases in number and average size of foci during 49 weeks, the total section area occupied by focal tissue was enhanced 10-fold without and 100-fold with PB treatment (not shown and Ref. 25). If compared to the 1.3-fold increment of liver mass during PB treatment (Fig. 2), these findings illustrate the growth advantage of focal cells over normal hepatocytes.

We have also calculated foci numbers per cm³ tissue and per total liver. The results shown in Table 3 support the conclusion that the number of detectable foci/liver did not change without PB but increased severalfold with PB treatment. For such calculations a spherical shape of foci is assumed. We felt that this condition was not met sufficiently at 49 weeks of PB treatment, where the calculation was not done.

When PB treatment was ended at 10 weeks, foci numbers rapidly declined back to levels in the group NNMTreatment → 0. Following withdrawal at 28 weeks, foci numbers also regressed but did not completely return to levels in the NNM → 0 group (Figs. 5 and 6b). The mean size of foci stopped increasing further after cessation of PB treatment and remained essentially un-

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Fig. 3. Morphological appearance of altered liver foci. a and b, NNM → 0, 42 weeks after NNM, H&E; a, clear cell focus, × 160; b, basophilic focus with tigroid hepatocytes, × 200; c, ground-glass liver focus, NNM → PB (10 weeks), H&E, × 200; note the sharp demarcation of the focus from the surrounding phenotypical normal tissue; d, GGT-positive liver focus with indistinct border, NNM → 0, 18 weeks after NNM; note the decreased stain for GGT towards the central vein (cv), × 160; e, GGT-positive liver focus with distinct border, NNM → PB, 34 weeks, × 100; f, GGT-positive liver focus with indistinct border, NNM → PB (28 weeks) → 0 (2 weeks), × 140.
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Fig. 4. Coincidence of expression of GGT with a second marker, cytochrome P-450-PB (a), glutathione-S-transferase B (b), and glycogen storage (c). Treatment, rats treated with NNM received basal diet for 8 weeks and then basal diet (NNM → 0) or PB (NNM → PB) for 2 weeks. □, foci with both markers; □, foci with GGT alone; □, foci with second marker alone; brackets beside columns; percentage of foci expressing GGT. Total number of foci analyzed is shown at the top of columns.

Table 2 Effect of PB on formation of liver tumors in NNM treated rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Neoplastic nodules</th>
<th>Carcinoma</th>
<th>Nodules + carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNM-0 (10-57 wk)</td>
<td>0</td>
<td>0</td>
<td>1/33*</td>
</tr>
<tr>
<td>NNM-PB (2-22 wk)</td>
<td>0</td>
<td>0</td>
<td>0/25</td>
</tr>
<tr>
<td>NNM-PB (28 wk)</td>
<td>4</td>
<td>1</td>
<td>5/7</td>
</tr>
<tr>
<td>NNM-PB (34 wk)</td>
<td>4</td>
<td>0</td>
<td>5/4</td>
</tr>
<tr>
<td>NNM-PB (49 wk)</td>
<td>1</td>
<td>2</td>
<td>3/6*</td>
</tr>
<tr>
<td>NNM-PB (28 wk)</td>
<td>2</td>
<td>0</td>
<td>2/5</td>
</tr>
<tr>
<td>NNM-0 (6 wk)</td>
<td>1</td>
<td>0</td>
<td>1/5</td>
</tr>
<tr>
<td>NNM-0 (10 wk)</td>
<td>0</td>
<td>1</td>
<td>1/6</td>
</tr>
</tbody>
</table>

* Tumor-bearing rats/total number of rats.

DNA Synthesis in Normal Liver and in Liver Foci. DNA synthesis in normal liver was very low, with approximately 0.1% of hepatocytes being labeled at any time point beyond 10 weeks (Fig. 7). Continuous PB treatment caused no increase, with the possible exception of the first time point (2 weeks). Obviously, the stimulatory effect of PB on DNA synthesis of normal hepatocytes, which has been noted before (9, 24), is limited to the initial stages of treatment. Our data, which were confirmed by biochemical assays (25), do not indicate decrease of DNA synthesis during prolonged exposure to PB, as reported by others (8, 27, 28). Thus, in our model there is no evidence of an antiproliferative (or “mitoinhibitory”) effect of PB on normal hepatocytes.

Hepatocytes in foci had much higher LIs than normal liver cells, i.e., between 1 and 2% during most of the experiment. Somewhat unexpectedly, PB treatment caused no further increase of LI at any time investigated. Taken together, our present (Fig. 7) and previous results (9, 29) indicate that PB initially increases DNA synthesis in foci but that this increase is short lived and disappears during continuous PB treatment.

After withdrawal of PB, no major changes occurred in DNA synthesis of normal hepatocytes. In foci the LIs did not decline; the tendency towards an increase was statistically not significant (Fig. 7).

Fig. 5. Size distribution of liver foci. GGT-positive foci were classified according to the number of cells/focus cross-section; the width of the size classes is 20 cells. The total number of foci/class/animal was normalized per cm²/tissue section; means of each animal group are given. Left, NNM only; center, NNM → PB; right and diagonal arrows, PB withdrawal; w, weeks after start of PB.

From the LIs obtained (Fig. 7b), we calculated the theoretical growth rate of the foci, assuming that no cell loss occurred. As seen in Fig. 8a, without PB treatment calculated foci growth rates were much higher than those found experimentally. Obviously, some cells disappeared from foci during the first months of the experiment. In contrast, in PB-treated rats foci growth rates that were calculated and were found experimentally agreed quite well (Fig. 8b). These results suggest that PB inhibited disappearance of focal cells and, thereby, induced enlargement of foci.

DNA Synthesis in Foci of Different Sizes. In the experiment shown in Fig. 7, large foci on average had no higher LIs than small ones (not shown). Because [3H]thymidine pulses result in low LIs with relatively high statistical variation, we have made use of another experiment (with identical treatments) in which [3H]thymidine was infused continuously for 2 weeks by means of osmotic minipumps. The LI of normal (nonfocal) hepatocytes was 3% without and 10% with PB treatment and the mean LIs in foci were 38 and 50%, respectively (29). Fig. 9 displays LIs of individual foci without or with PB treatment.

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<td>0</td>
<td>0</td>
<td>1/33*</td>
</tr>
<tr>
<td>NNM-PB (2-22 wk)</td>
<td>0</td>
<td>0</td>
<td>0/25</td>
</tr>
<tr>
<td>NNM-PB (28 wk)</td>
<td>4</td>
<td>1</td>
<td>5/7</td>
</tr>
<tr>
<td>NNM-PB (34 wk)</td>
<td>4</td>
<td>0</td>
<td>5/4</td>
</tr>
<tr>
<td>NNM-PB (49 wk)</td>
<td>1</td>
<td>2</td>
<td>3/6*</td>
</tr>
<tr>
<td>NNM-PB (28 wk)</td>
<td>2</td>
<td>0</td>
<td>2/5</td>
</tr>
<tr>
<td>NNM-0 (6 wk)</td>
<td>1</td>
<td>0</td>
<td>1/5</td>
</tr>
<tr>
<td>NNM-0 (10 wk)</td>
<td>0</td>
<td>1</td>
<td>1/6</td>
</tr>
</tbody>
</table>

* Tumor-bearing rats/total number of rats.

Three additional rats had small macroscopically visible tumors, from which no histological diagnoses were obtained.

changed; after 18 or 21 weeks, respectively, the difference from the NNM → 0 group had disappeared, apparently because of catch-up growth in the latter (Fig. 6a).

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Fig. 6. Time course of increases in average size and number of liver foci. Treatment, symbols, and number of animals, see Figs. 1 and 2. a, average foci size; b, foci number. Means are given; vertical bars, SD. *, P< 0.05, NNM → PB versus NNM → 0 or NNM → PB → 0 killed at the same time point; Φ, P< 0.05, NNM → PB → 0 versus NNM → PB (time of PB withdrawal).

Table 3: Foci number per cm² and per liver

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Foci no./cm²</th>
<th>Foci no./liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NNM → 0 (8 wk) → 0</td>
<td>0 wk 4</td>
<td>308 ± 97</td>
<td>1.966 ± 658</td>
</tr>
<tr>
<td></td>
<td>10 wk 3</td>
<td>316 ± 122</td>
<td>1.561 ± 587</td>
</tr>
<tr>
<td></td>
<td>28 wk 5</td>
<td>469 ± 185</td>
<td>2.337 ± 1.135</td>
</tr>
<tr>
<td></td>
<td>34 wk 2</td>
<td>291</td>
<td>1.751</td>
</tr>
<tr>
<td></td>
<td>49 wk 3</td>
<td>393 ± 248</td>
<td>2.078 ± 1.256</td>
</tr>
<tr>
<td>NNM → 0 (8 wk) → PB</td>
<td>2 wk 4</td>
<td>481 ± 271</td>
<td>4.066 ± 2.081</td>
</tr>
<tr>
<td></td>
<td>4 wk 4</td>
<td>934 ± 420</td>
<td>7.473 ± 2.920</td>
</tr>
<tr>
<td></td>
<td>10 wk 7</td>
<td>738 ± 236 ²</td>
<td>4.824 ± 1.667 ²</td>
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<tr>
<td></td>
<td>28 wk 7</td>
<td>786 ± 459</td>
<td>7.085 ± 2.742</td>
</tr>
<tr>
<td></td>
<td>34 wk 5</td>
<td>1.202 ± 194 ³</td>
<td>9.820 ± 2.545 ³</td>
</tr>
<tr>
<td>Experiment 2 ⁴</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>NNM → 0 (11 wk)</td>
<td>4</td>
<td>333 ± 120</td>
<td>2.228 ± 471</td>
</tr>
<tr>
<td>NNM → 0 (13 wk)</td>
<td>2</td>
<td>356</td>
<td>1.915</td>
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<tr>
<td>NNM → 0 (11 wk) → PB</td>
<td>2 wk 5</td>
<td>975 ± 422</td>
<td>8.240 ± 3.627</td>
</tr>
</tbody>
</table>

* P< 0.01.
² P< 0.05 NNM → PB versus NNM → 0.
³ Results from an independent experiment performed with identical treatment conditions.

There was no positive correlation between size of foci and DNA synthesis activity. This finding supports the notion that, in the experimental stage investigated, the rate of DNA synthesis cannot be the only determinant of foci growth.

Another important result of this experiment is the following: PB increased the number of foci expressing GGT by a factor of 4 in this particular experiment (Table 3, experiment 2). Had this increase occurred by proliferation of single (initiated) cells, numerous foci should show LIs of 100%. However, only a single 4-cell focus had this LI. This finding provides a proof that PB caused multiplication of foci in the present experiment by induction of GGT expression in preexistent but GGT-negative foci.

Phenotype Expression and Cell Death in Foci. Two mechanisms conceivably could counterbalance the effect of the high replicative activity in foci, namely (a) cell death or (b) failure to express GGT and other phenotypic markers, so that foci cells could be discriminated from normal hepatocytes. It was noted that the intensity of GGT staining was much weaker in the majority of foci in the absence of PB treatment and many foci were not sharply demarcated from the surrounding parenchyma (Fig. 3, d and f). In an attempt to quantify the intensity of phenotypic expression, we have recorded for each focus whether it had distinct (DB) or indistinct (INDB) borders. The results are shown in Figs. 10 and 11. Four points should be noted. (a) INDB foci on average were smaller than DB foci. (b) This effect was particularly pronounced at the end of the experimental period, suggesting that DB foci grew while INDB foci did not or grew only little. (c) PB decreased the percentage of INDB foci and further enhanced growth of DB foci (Fig. 10). (d) The effect of PB was reversible after cessation of treatment (Fig. 11a). These results suggest that the strength of
phenotypic expression is a determinant of the detectable size of the foci and of their growth rate; growth and phenotypic expression of foci induced by PB appear to be correlated.

Next we studied the incidence of DNA synthesis and of cell death by apoptosis in INDB and DB foci. The two classes of foci showed no difference in the rate of DNA synthesis with or without PB treatment (Fig. 11b), but the number of apoptotic bodies in INDB foci exceeded that in DB foci by a factor of 4 (Fig. 11c). PB treatment did not alter the incidence of apoptosis in the two types of foci but, due to the shift in favor of DB foci (Fig. 11a), the overall incidence of apoptosis in the whole population of foci was much lower than without PB, a result consistent with previous data (23). After cessation of PB feeding, apoptotic numbers increased in both classes of foci, but still at two of three time points INDB foci contained considerably more ABs than DB foci. It was also noted that ABs were located predominantly in those areas of foci exhibiting weak expression of phenotypic alterations. These results suggest that low intensity of phenotypic expression by hepatocytes in foci is associated with an enhanced probability to undergo death. Therefore, in INDB foci cell death appears to counterbalance the enhanced rate of cell replication.

**DISCUSSION**

The present study was designed to follow the effect of the model tumor promoter PB on the development of hepatocellular carcinoma from a stage as early as possible after initiation. That our protocol produced an early type of preneoplastic lesions is suggested by the following findings: (a) the initial predominance of clear cell foci, (b) the relative rarity and small size of histologically detectable lesions, (c) the unstable phenotypic expression in most of the lesions (see below), and (d) the late appearance of tumors without PB treatment. Continuous PB treatment significantly accelerated the development of these early lesions into neoplastic nodules and hepatocellular carcinomas. A diagram depicting a schematic concept of tumor promotion in the liver as derived from this and previous studies is shown in Fig. 12.

The acceleration of tumor development by PB was associated with three characteristic effects on hepatocellular foci, namely (a) changes in phenotypic appearance, (b) increases in number, and (c) accelerated enlargement of detectable foci. Phenotype changes included a shift from clear to eosinophilic and basophilic appearance, increased coincidence of expression of various markers by different foci, stronger expression of GGT in many individual foci, and the appearance of distinct borders between focal and surrounding tissue. Thus, the expression of the altered phenotype of foci is weak and incomplete early after initiation and can be enhanced by PB (see Fig. 12). These observations confirm and extend previous findings with different initiation-promotion protocols (4, 7, 30-34). The second effect of PB, the increase in foci number, was not due to formation of new foci, at least not during the first 2 weeks of PB treatment. Therefore, the increase in foci number, at least during early weeks, is not a true one, but the stronger expression of phenotypic changes renders more foci detectable (see Fig. 12).
animals are not identical. This could complicate comparisons in this focus. Promotion leads to expression of phenotypic alteration, decrease of cell death, and hence rapid growth of this type of focus. The mechanism of foci enlargement turned out to be more complex than anticipated. Although cell replication, as reflected by DNA synthesis, was always about 10-fold higher in foci than in normal liver, as found before (9), it cannot be the only determinant of foci growth for the following arguments: (a) the lack of foci enlargement during early months after NNM treatment, (b) the lack of correlation between size and LI of individual (early) foci, and (c) the frequent occurrence of apoptosis in foci.

Quantitative assessment of the importance of apoptosis as a determinant of foci growth and regression requires, in addition to data on AB incidence in histological sections, information on the duration of apoptosis. This has recently been determined to be 3 h in normal liver and is probably similar in foci (35). From the present data (Fig. 11) and the procedure described (35), we can calculate apoptotic rates of approximately 0.8% in INDB foci in groups NNM → PB per 3-h interval. Rates per day may well be higher. Therefore, it seems justified to assume that in INDB foci apoptosis indeed counterbalances cell proliferation; hence, phenotypic remodeling would not be a major cause of cell loss in these foci.

Instead, by increased cell replication PB thus seems to promote foci growth by inhibition of cell loss due to apoptosis and to phenotypic remodeling (present study and Refs. 4, 6, 7, 23, 36, and 37) (see Fig. 12). As a result, the high proliferative activity in foci could manifest itself. The failure of PB to increase cell proliferation in foci should not be generalized to other promoters. In a parallel study α-hexachlorocyclohexane produced a clear tendency to increased DNA synthesis in foci, resulting in even more rapid foci enlargement.

A further aim of the present study was to investigate the question of reversibility of tumor promotion. Obviously, the effects of PB on phenotypic expression and on cell number in foci need to be considered separately. The former appears to be largely reversible, at least during early stages, as suggested by the changes seen in H&E-stained sections and also by the decline of foci numbers back to control level; this decline was incomplete after 28 weeks of treatment. Similar findings were made before (30). On the other hand, the increase in cell number does not appear to be as readily reversible (30, 36). The frequency of apoptosis in foci is enhanced after PB withdrawal. From the data in Fig. 11 and Ref. 35, we can calculate apoptotic rates of 1.5-3%/3-h interval. Although the rates per day cannot yet be derived from this figure due to a possible circadian fluctuation (37), it seems likely that in the present experiments cell death rates exceeded replication rates in the average focus after PB withdrawal. This supports the view that to some extent tumor promotion may be reversible in the liver.

Of particular interest is the apparent association between loss of strong phenotypic expression and increase of apoptosis after
PB withdrawal. A similar observation has been made by Garcea et al. (38), during regression of foci and nodules after treatment with S-adenosylmethionine. On the other hand, loss of some phenotypic markers such as GGT in foci, as occurs during treatment with peroxisome inducers, is not associated with enhanced cell death (39). The situation in remodeling foci after promoter withdrawal, therefore, seems to resemble "functional atrophy" in normal liver such as during starvation, when many proteins are degraded and the incidence of apoptosis is enhanced (37). Likewise, hyperplastic-hyperfunctional liver after mitogen treatment degrades enzymes and eliminates cells when the mitogen is withdrawn (23, 37). PB effects on foci thus resemble the effects of functional stimuli on their target organ.

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DNA Synthesis, Apoptosis, and Phenotypic Expression as Determinants of Growth of Altered Foci in Rat Liver during Phenobarbital Promotion

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