Reversibility of promoter induced hepatic focal lesion growth in mice

Kyle L.Kolaja¹*, Donald E.Stevenson²,
Earl F.Walborg Jr² and James E.Klaunig¹,³

¹Division of Toxicology, Department of Pharmacology and Toxicology, Indiana University School of Medicine, 1001 Walnut Street MRF 003, Indianapolis, IN 46202 and ²Dermigen Inc., Smithville, TX, USA

*To whom correspondence should be addressed

The effect of cessation of phenobarbital and dieldrin treatment on hepatic focal lesion growth in male B6C3F1 mice was investigated. Following induction of lesions by diethylnitrosamine, mice were placed on control NIH-07 diet (control diet) or NIH-07 diet containing either dieldrin (10.0 mg/kg diet) or phenobarbital (500 mg/kg diet). Mice were sacrificed after 30 and 60 days of dietary treatment. Two additional groups of mice were fed either the dieldrin- or phenobarbital-containing diet for 30 days followed by feeding of NIH-07-only diet for an additional 30 days. The effect of treatment and removal of dieldrin or phenobarbital on lesion growth was examined by measuring both the number of focal lesions per liver and the relative volume of focal lesions. In addition, the rate of cell proliferation and programmed cell death in focal lesion growth was investigated by examining DNA synthesis and apoptosis in the focal lesions. Dietary dieldrin or phenobarbital increased the number of focal lesions and the focal lesion volume. In both dieldrin- and phenobarbital-treated mice, an increased number of eosinophilic lesions were seen. The focal lesion volume was increased in both eosinophilic and basophilic lesions. Dieldrin and phenobarbital treatment also increased the DNA synthetic labeling index in both eosinophilic and basophilic lesions. Removal of dieldrin or phenobarbital from the diet after 30 days of promoter treatment decreased the total number and volume of hepatic focal lesions. The labeling index of the focal lesions was also decreased in these mice. At the terminal sacrifice, the percentage of apoptotic cells in focal lesions was higher in mice fed dieldrin- or phenobarbital-containing diets for the entire 60 days than in mice returned to control diet for the last 30 days. Eosinophilic lesions were more dependent on the presence of a promoting stimulus than the basophilic lesions. These data indicate that induction and maintenance of the growth of some preneoplastic lesions in the mouse may be dependent upon continuous tumor promoter treatment.

Introduction

Hepatocarcinogenesis is a multi-stage process. It is characterized by a step-wise alteration of a normal cell to a preneoplastic intermediate that has the ultimate potential to become a malignant neoplastic cell. Prior to the onset of hepatic neoplasia, putative preneoplastic focal lesions have been observed (1,2). These morphologically distinct focal lesions exhibit altered gene expression and function when compared to normal, surrounding liver and appear to be precursors to malignant hepatocellular carcinoma, albeit, only a minority of these focal lesions will develop into hepatic cancer (1,2). Therefore, investigations into the growth of early preneoplastic lesions may aid in understanding those functions, genetic and epigenetic, that influence their progressive development into more autonomous lesions.

Chronic exposure to selected nongenotoxic chemicals results in the onset of hepatocellular cancer (3-9). These agents do not interact with DNA directly and their mechanism of hepatocarcinogenic action is considered to be epigenetic (10). Studies by several groups have shown that continuous exposure to nongenotoxic hepatocarcinogens after treatment with an initiating carcinogen results in the increased growth of hepatic focal lesions (3,11,12,19-25) suggesting that these compounds induce hepatic cancer by functioning at the stage of tumor promotion (3-5).

A number of species-specific nongenotoxic hepatocarcinogenic organochlorine pesticides (aldrin, dieldrin, heptachlor, and endosulfan) have been proposed to be hepatic tumor promoters (10-12). Chronic exposure to dieldrin results in hepatic cancer in mice but not rats, hamsters, dogs, or monkeys (13-17). Similarly, recent studies have shown that dieldrin promoted the growth of preformed hepatic focal lesions in mice but not rats (18). In contrast, the barbiturate phenobarbital (PB*), a nongenotoxic hepatocarcinogen in both mice and rats, has been shown to promote the growth of preneoplastic focal lesions in both mice and rats (19-25). These observations indicate that these nongenotoxic agents influence the growth of hepatic focal lesions by mechanisms that are species-specific.

In a variety of rat hepatocarcinogenesis models, it has been demonstrated that early preneoplastic lesions are reversible and disappear upon removal of the promoting stimulus (26-29). Chronic feeding of 5 mg diethylnitrosamine (DEN)/kg body weight per day to rats was cytotoxic and resulted in the formation of focal lesions that disappeared after cessation of treatment (26). Three cycles of 2-acetaminofluorene (2-AAF; 3 weeks feeding of 0.06% 2-AAF then 1 week on basal diet) resulted in the formation of macroscopic nodules that disappear a few months after the last cycle of treatment (27). DEN treatment of partial hepatectomized rats followed by continuous treatment by PB resulted in the formation of altered hepatic foci that disappeared upon PB removal (28). Also, DEN (200 mg/kg body weight) followed by partial hepatectomy and promoted with 2-AAF resulted in the formation of visible hyperplastic hepatic nodules (29). Subsequent removal of 2-AAF resulted in a decrease in the number and size of hyperplastic nodules. These studies suggest that some altered hepatic foci in rats may require the continuous presence of a promoting agent to prevent their regression.
A fundamental question regarding nongenotoxic induced-hepatocarcinogenesis is the stability and persistence of hepatic focal lesion growth following the removal of promoting stimuli. The studies mentioned above suggest some rat hepatic focal lesions require the presence of a promoting agent. Much less is known about the growth characteristics of mouse hepatic lesions. Lipsky reported that cessation of safrole treatment to mice after 52 weeks of exposure resulted in a decreased number of altered hepatic foci in mice (30). In addition, cessation of hexachlorocyclohexane treatment resulted in the loss of hyperplastic areas and focal lesions (31). Reuben suggested that the increased incidence of hepatocarcinogenicity in mice compared to rats may be attributed to an inability of the surrounding hepatocytes to respond to nongenotoxic agents (32).

Biological differences between early focal lesions in mice and rats may respond differently to nongenotoxic treatment and removal was examined by investigating the modulation of local lesion growth after dieldrin and PB on the modulation of local lesion growth after dieldrin and PB. Dieldrin did not appear to inhibit lesion growth and the effect of removal of dieldrin and PB on the growth of focal lesions and the effect of removal of dieldrin and PB on the growth of focal lesions (18). Furthermore, dieldrin concentration of hepatic focal lesions was determined by using two different methods (33,35).

Phenotypic diversity of focal preneoplastic lesions has been observed in mice and rats following treatment with different nongenotoxic hepatocarcinogens (36-38). PB treatment has been shown to enhance the growth of focal lesions that are morphologically different (39,40) and PB inhibits apoptosis, programmed cell death, preferentially in the nonspecific in the nonspecific focal lesions (25). Previously, we have shown that dieldrin nonselectively promotes the growth of basophilic and eosinophilic hepatic focal lesions (18). Furthermore, dieldrin did not appear to inhibit apoptosis in either basophilic or eosinophilic focal lesions (18). These studies indicate that nongenotoxic hepatocarcinogens like dieldrin and PB preferentially enhance the growth of focal hepatic lesions by different mechanisms.

The present study investigated the differential effects of dietary PB and dieldrin in DEN-induced mouse hepatic focal lesion growth and the effect of removal of dieldrin and PB on focal preneoplastic liver lesions in DEN-initiated B6C3F1 mice. Specifically, we examined the changes in number and volume of hepatic focal lesions after treatment and removal of two known promoting agents (PB and dieldrin). Furthermore, the modulation of focal lesion growth after dieldrin and PB treatment and removal was examined by investigating the incidence of apoptosis and DNA synthesis in distinct focal liver lesions after the removal of the promoting agent.

### Materials and methods

#### Chemicals

DEN and PB were purchased from Sigma Chemical Co., St Louis, MO. Dieldrin (99%) was provided by Demingens, Inc., Smithville, TX. NIH-07 diets containing 100 mg dieldrin kg diet and 500 mg PB kg diet were formulated by Dey's Bethlehem PA. Concentrations of test compounds were verified as described previously (39).

#### Animals and experimental design

Three-week-old male B6C3F1 mice were purchased from Harlan Sprague-Dawley Co. (Indianapolis, IN). Mice were housed in polyethylene cages in a AAALAC-accredited animal facility. All mice were maintained in accordance with the NIH-Guide for the Care and Use of Laboratory Animals. All mice received NIH-07 diet in pelleted form and water ad libitum during a 1-week acclimation period. At the conclusion of the acclimation period, B6C3F1 mice were given two 35 mg DEN/kg body weight ip injections per week for 8 weeks. When focal lesions were apparent (after 3 months), the mice were randomly placed into one of the following treatment groups (five mice per group): Group A: 30 days on NIH-07 diet (Control), Group B: 30 days on 100 mg dieldrin kg NIH-07 diet, Group C: 30 days on 500 mg PB kg NIH-07 diet, Group D: 30 days on NIH-07 diet (Control), Group E: 60 days on 100 mg dieldrin kg NIH-07 diet, Group F: 60 days on 500 mg PB kg NIH-07 diet. Group G: 30 days on 100 mg dieldrin NIH-07 diet then 30 days on NIH-07 diet. Group H: 30 days on 500 mg PB kg NIH-07 diet then 30 days on NIH-07 diet. Osmitol micropumps (model 2001) Alzet Co., Palo Alto, CA) containing bromodeoxyuridine (BrdU), 16 mg/ml of PBS were surgically implanted into mice from each treatment group 7 days prior to each sacrifice date. At sampling, animals were humanely killed by cervical dislocation, weighed and necropsied. The livers were resected, weighted and examined for the presence of grossly visible lesions. The livers were then separated by lobe and sectioned into 1.2 mm strips. Strips of liver from each block were fixed in formalin for 48-72 h and then embedded in paraffin. A total of three paraffin blocks per animal were produced. Serial sections from each block were stained with hematoxylin and eosin or submitted to immunohistochemical detection of BrdU (see below). Hepatic preneoplastic lesions were classified as previously described (40). Briefly, individual focal lesions were classified as follows.

- **Hepatocytes in eosinophilic focal lesions**: can be either smaller or larger than normal surrounding hepatocytes, however, in this study, they were larger than neighboring hepatocytes. The cytoplasm has a distinct granular appearance compared to adjacent hepatocytes (40).

- **Hepatocytes in basophilic focal lesions**: can be either smaller or larger than normal hepatocytes, in this study, however, they were smaller than normal hepatocytes. The cytoplasm of hepatocytes in basophilic, focal lesions is distinctly basophilic compared to adjacent hepatocytes.

- **Clear focal lesions** as well as accumulated focal lesions consist of a clear, ground glass cytoplasm. The cells in these focal lesions tend to be larger than normal hepatocytes.

- Both adenomas and carcinomas exist as distinct nodules which compress adjacent parenchyma and can bulge from the liver surface. Adenomas are composed of cells that are well differentiated, forming a solid nodule that resembles normal liver. No invasion of adjacent parenchyma but compression of the surrounding hepatocytes is observed on at least three sides of the lesion (41).

- BrdU immunohistochemistry was performed according to previously published methods with minor modifications (42). Hepatocytes that had incorporated BrdU were easily identified by the accumulation of red pigment within the nucleus as compared to the counterstained blue nuclei of nonlabeled cells. A stretch of duodenum from each animal was included on all slides to examine proper staining and incorporation of BrdU into the tissues. In focal lesions, all hepatocytes were scored. Labeling index (the percentage of cells that stained with antibody BrdU-labeled nuclei by an IBM computer).

- **Microscopic examination of hepatocytes and eosin stained slides was used to quantify the incidence of apoptosis. Apoptosis in the liver was defined and quantitated as previously described (43). In addition, this manner of identification of apoptotic cells was further validated by using two different methods**.

#### Table 1. Body weights and relative liver weights in dieldrin and phenobarbital treated B6C3F1 mice

<table>
<thead>
<tr>
<th>Group Treatment</th>
<th>Body weight (g)</th>
<th>Relative liver weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH-07 control (30 days)</td>
<td>29.2 ± 0.7</td>
<td>7.1 ± 0.3</td>
</tr>
<tr>
<td>100 mg dieldrin kg diet (30 days)</td>
<td>28.1 ± 1.0</td>
<td>8.0 ± 0.7</td>
</tr>
<tr>
<td>500 mg phenobarbital kg diet (30 days)</td>
<td>31.7 ± 2.5</td>
<td>9.4 ± 0.9</td>
</tr>
<tr>
<td>NIH-07 control (60 days)</td>
<td>29.4 ± 1.4</td>
<td>7.4 ± 0.9</td>
</tr>
<tr>
<td>100 mg dieldrin kg diet (60 days)</td>
<td>29.4 ± 2.2</td>
<td>10.4 ± 1.8</td>
</tr>
<tr>
<td>500 mg phenobarbital kg diet (60 days)</td>
<td>29.4 ± 1.7</td>
<td>10.0 ± 0.7</td>
</tr>
<tr>
<td>NIH-07 control (90 days)</td>
<td>30.5 ± 2.1</td>
<td>7.3 ± 1.0</td>
</tr>
<tr>
<td>100 mg dieldrin kg diet (90 days)</td>
<td>30.0 ± 1.2</td>
<td>7.3 ± 0.6</td>
</tr>
</tbody>
</table>

Values represent the mean ± SD of the body weight and the relative liver weight (body weight to relative liver weight ratio in dieldrin 100 mg kg or phenobarbital 500 mg kg treated B6C3F1 mice initiated with diethylnitrosamine (two 35 mg kg injections per week for 8 weeks).

#### Statistical analysis

From treatment Group A 6 Group F 6 Group H 6 Group I determined by ANOVA (p 0.05).
Table II. Hepatic focal lesion number per liver in dieldrin and phenobarbital treated B6C3F1 mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Basophilic</th>
<th>Eosinophilic</th>
<th>Clear</th>
<th>All foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NIH-07 control (30 days)</td>
<td>105±41</td>
<td>194±95</td>
<td>114±62</td>
<td>407±128</td>
</tr>
<tr>
<td>B</td>
<td>10.0 mg dieldrin/kg diet (30 days)</td>
<td>195±77</td>
<td>449±145b</td>
<td>80±51</td>
<td>705±212b</td>
</tr>
<tr>
<td>C</td>
<td>500 mg phenobarbital/kg diet (30 days)</td>
<td>831±42</td>
<td>521±87b</td>
<td>189±98</td>
<td>779±121b</td>
</tr>
<tr>
<td>D</td>
<td>NIH-07 control (60 days)</td>
<td>146±56</td>
<td>194±115</td>
<td>98±69</td>
<td>431±206</td>
</tr>
<tr>
<td>E</td>
<td>10.0 mg dieldrin/kg diet (60 days)</td>
<td>195±77</td>
<td>744±227b</td>
<td>119±121</td>
<td>1095±316b</td>
</tr>
<tr>
<td>F</td>
<td>500 mg phenobarbital/kg diet (60 days)</td>
<td>206±67</td>
<td>467±109b</td>
<td>120±42</td>
<td>696±104b</td>
</tr>
<tr>
<td>G</td>
<td>30 days 10.0 mg dieldrin/kg diet then 30 days NIH-07 control diet</td>
<td>216±59</td>
<td>276±79b</td>
<td>20±25</td>
<td>504±119b</td>
</tr>
<tr>
<td>H</td>
<td>30 days 500 mg phenobarbital/kg diet then 30 days NIH-07 control diet</td>
<td>128±69</td>
<td>250±57b</td>
<td>24±21</td>
<td>95±127b</td>
</tr>
</tbody>
</table>

Values represent the mean ± SD of the number of hepatic focal lesions per liver in dieldrin (10.0 mg/kg) or phenobarbital (500 mg/kg) treated B6C3F1 mice initiated with diethylnitrosamine (two 35 mg/kg injections per week for 8 weeks). Statistical significance from treatment Group A, Group D, Group E or Group F determined by ANOVA (P < 0.05).

Table III. Hepatic focal volume in dieldrin and phenobarbital treated B6C3F1 mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Basophilic</th>
<th>Eosinophilic</th>
<th>Clear</th>
<th>All foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NIH-07 control (30 days)</td>
<td>1.08±0.52</td>
<td>1.36±1.15</td>
<td>0.49±0.40</td>
<td>2.80±1.43</td>
</tr>
<tr>
<td>B</td>
<td>10.0 mg dieldrin/kg diet (30 days)</td>
<td>1.75±0.13</td>
<td>3.05±1.23a</td>
<td>0.24±0.18</td>
<td>4.89±1.99b</td>
</tr>
<tr>
<td>C</td>
<td>500 mg phenobarbital/kg diet (30 days)</td>
<td>0.57±0.26</td>
<td>2.61±1.04</td>
<td>0.39±0.34</td>
<td>3.50±1.40</td>
</tr>
<tr>
<td>D</td>
<td>NIH-07 control (60 days)</td>
<td>0.51±0.37</td>
<td>1.25±0.89</td>
<td>0.22±0.21</td>
<td>1.96±1.25</td>
</tr>
<tr>
<td>E</td>
<td>10.0 mg dieldrin/kg diet (60 days)</td>
<td>1.70±0.40b</td>
<td>4.68±0.51b</td>
<td>0.29±0.22</td>
<td>6.48±0.72b</td>
</tr>
<tr>
<td>F</td>
<td>500 mg phenobarbital/kg diet (60 days)</td>
<td>1.42±0.40b</td>
<td>3.10±1.02b</td>
<td>0.24±0.14</td>
<td>5.20±0.87b</td>
</tr>
<tr>
<td>G</td>
<td>30 days 10.0 mg dieldrin/kg diet then 30 days NIH-07 control diet</td>
<td>1.58±0.50b</td>
<td>1.74±1.06b</td>
<td>0.02±0.05</td>
<td>3.30±1.50b</td>
</tr>
<tr>
<td>H</td>
<td>30 days 500 mg phenobarbital/kg diet then 30 days NIH-07 control diet</td>
<td>1.08±0.66</td>
<td>2.47±1.50</td>
<td>0.07±0.05</td>
<td>3.57±1.01d</td>
</tr>
</tbody>
</table>

Values represent the mean ± SD of the hepatic focal volume percentage (%) in dieldrin (10.0 mg/kg) or phenobarbital (500 mg/kg) treated B6C3F1 mice initiated with diethylnitrosamine (two 35 mg/kg injections per week for 8 weeks). Statistical significance from treatment Group A, Group D, Group E or Group F determined by ANOVA (P < 0.05).

methodologies: the Apoptag staining kit (Oncor Inc., Gaithersburg, MD) and a fluorescence microscopy method (46). The number of apoptotic hepatocytes (at any morphological phase) in focal lesions was divided by the total number of hepatocytes in that lesion and multiplied by 100 to achieve an apoptotic index.

Statistics

Statistical difference (P < 0.05) from control values for all data was determined by using ANOVA followed by a Dunnett's post hoc (47).

Results

The effect of dieldrin treatment and cessation of treatment on body weight and relative liver weight (liver to body weight ratio) is shown in Table I. Treatment with dieldrin (30 days; Group E) resulted in an increased relative liver weight. Removal of dieldrin from the diet (Group G) was accompanied by return of the relative liver weight to that of mice on the control diet (Group D). The effect of PB treatment and cessation of treatment on body weight and relative liver weight (liver to body weight ratio) is also shown in Table I. PB treatment for 30 or 60 days (Groups C and F, respectively) resulted in increased relative liver weights compared to control (Groups A and D, respectively). Removal of PB from the diet (Group H) decreased the relative liver weight, when compared to continuously fed mice (Group F). No treatment significantly affected body weight at any time point examined (Table I).

The number of focal lesions per liver in dieldrin-treated mice is shown in Table II. Exposure to dieldrin for 30 or 60 days (Groups B and E, respectively) increased the total number of focal lesions per liver. Eosinophilic lesions were preferentially effected as reflected by their increased number. Cessation of dieldrin treatment (Group G) significantly decreased the total number of focal lesions per liver. This decreased total number of focal lesions per liver was due to a decrease in the number of eosinophilic lesions. Table II also shows the number of focal lesions per liver in PB-treated mice. After 30 and 60 days of dietary exposure to PB (Groups C and F, respectively), an increase in the total number of focal lesions per liver was seen. Similar to dieldrin treated mice, the number of eosinophilic lesions was preferentially increased. Cessation of PB treatment (Group H) significantly decreased the total number of focal lesions per liver. In a similar manner to dieldrin-treated mice, this decreased total number of focal lesions per liver in PB-treated mice was due to a decrease in the number of eosinophilic lesions. The number of basophilic or clear focal lesions was not affected by any treatment at any time point examined (Table II).

Table III shows the effect of dieldrin treatment and withdrawal on the focal lesion volume, expressed as percentage of total liver volume. Treatment with dieldrin for 30 and 60 days (Groups B and E, respectively) was accompanied by an increase in focal lesion volume. After 30 days of dieldrin treatment (Group B), the volume occupied by eosinophilic focal lesions was increased. After 60 days of dieldrin treatment (Group E), the hepatic focal volume of both basophilic and eosinophilic lesions was increased. Cessation of dietary treatment of dieldrin (Group G) was accompanied by a reduction in the volume of hepatic focal lesions (compare Groups G and E). Removal of dieldrin (Group G) from the diet decreased the volume occupied by eosinophilic lesions, but had no significant effect on any other lesion type examined. In fact, the volume of basophilic lesions in mice in Group G (dieldrin discontinued after 30 days) was not significantly different from that of mice fed dieldrin for 30 or 60 days (Groups B and E).
The effect of PB treatment and cessation on the focal lesion volume is also shown in Table III. PB treatment (30 days; Group C) increased the focal lesion volume. Despite the increased total focal volume, no significant increases in the hepatic focal volumes of any specific classes of foci were detected. However, after 60 days, PB treatment (Group F) significantly increased the volume in both basophilic and eosinophilic lesions. Cessation of dietary treatment of PB (Group H) decreased the volume of total focal lesions, compared to mice continuously fed PB (Group F). However, the decrease could not be attributed to a specific class of lesions (Table III).

The effect of dieldrin treatment and cessation on the DNA labeling index of focal lesions is shown in Table IV. After 30 and 60 days of dieldrin treatment (Groups B and E, respectively), an increase in the focal DNA labeling index was observed. After 30 or 60 days of treatment, dieldrin treatment significantly increased the labeling index of basophilic and eosinophilic lesions. Cessation of dietary treatment of dieldrin (Group G) had no observed effect upon the labeling index of all focal lesions, perhaps due to high interanimal variability. However, removal of dieldrin from the diet significantly decreased the labeling index of basophilic and eosinophilic lesions. Table IV also shows the effect of PB treatment and cessation on the focal lesion DNA labeling index. PB treatment for 30 days (Group C) increased the total focal DNA labeling index. After 30 days of treatment, PB treatment specifically increased the labeling index of basophilic lesions. However, after 60 days of treatment (Group F), PB significantly increased the labeling index of both basophilic and eosinophilic foci. Removal of PB from the diet (Group H) had no observed effect upon the labeling index of all focal lesions, perhaps due to high interanimal variability. However, removal of PB did decrease the labeling index of basophilic foci (Table IV).

For comparison, the effect of promoter treatment and cessation on the DNA labeling index in normal surrounding liver is shown in Table V. Dieldrin treatment (Groups B and E) increased the labeling index of normal liver. Subsequent removal of dieldrin from the diet (Group G) decreased the labeling index of normal liver. In contrast, no increase in phenobarbital treated mice was observed at either time point.

The effect of dieldrin treatment and cessation on the incidence of apoptosis in focal lesions is shown in Table VI. Dieldrin treatment (Groups B and E) had no effect on the incidence of apoptosis in focal lesions. Regardless of the lack of dieldrin-induced inhibition of apoptosis in foci, cessation of dieldrin (Group G) increased the incidence of apoptosis in foci (Figure 1). This increase in apoptosis in mice formerly fed dieldrin occurred in both eosinophilic and basophilic hepatic foci. Table VI also shows the effect of PB treatment and cessation on the incidence of apoptosis in focal lesions. In contrast, to the data mentioned above for dieldrin-treated foci, treatment with PB (30 and 60 days, Groups C and F) significantly decreased the incidence of apoptosis in hepatic foci and this decrease occurred in both basophilic and eosinophilic foci. Similar to dieldrin-treated mice, cessation of PB (Group H) drastically increased the incidence of apoptosis in focal lesions. The increased incidence of apoptosis observed after cessation of PB treatment occurred in both eosinophilic and basophilic hepatic foci (Table VI).

The incidence of apoptosis in normal, surrounding liver is shown in Table VII. PB treatment (Groups C and F) decreased the incidence of apoptotic hepatocytes. In contrast, dieldrin treatment (Groups C and F) had no measurable effect on the incidence of apoptosis in normal surrounding non-focal hepatocytes. However, subsequent removal of either dieldrin (Group G) or PB (Group H) increased the incidence of apoptosis in non-focal hepatocytes.

**Discussion**

This study demonstrated that feeding of dieldrin or PB to B6C3F1 mice stimulates the growth of DEN-induced hepatic foci. Eosinophilic lesions in particular showed an increase in their volume.
Reversibility of tumor promotion

Table VI. Incidence of focal hepatocyte apoptosis in dieldrin and phenobarbital treated B6C3F1 mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Basophilic</th>
<th>Eosinophilic</th>
<th>Clear</th>
<th>All foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NIH-07 control (30 days)</td>
<td>1.01±0.38</td>
<td>1.29±0.43</td>
<td>0.72±0.87</td>
<td>1.04±0.39</td>
</tr>
<tr>
<td>B</td>
<td>10.0 mg dieldrin/kg diet (30 days)</td>
<td>1.16±0.53</td>
<td>0.78±0.39</td>
<td>ND</td>
<td>0.87±0.36</td>
</tr>
<tr>
<td>C</td>
<td>500 mg phenobarbital/kg diet (30 days)</td>
<td>0.17±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.20±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>0.21±0.25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>D</td>
<td>NIH-07 control (60 days)</td>
<td>1.09±0.36</td>
<td>1.07±0.38</td>
<td>ND</td>
<td>1.02±0.31</td>
</tr>
<tr>
<td>E</td>
<td>10.0 mg dieldrin/kg diet (60 days)</td>
<td>0.98±0.64</td>
<td>0.82±0.74</td>
<td>0.83±0.29</td>
<td>1.27±0.38</td>
</tr>
<tr>
<td>F</td>
<td>500 mg phenobarbital/kg diet (60 days)</td>
<td>0.52±0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.38±0.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.18±0.21</td>
<td>0.65±0.20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>G</td>
<td>30 days 10.0 mg dieldrin/kg diet then 30 days NIH-07 control diet</td>
<td>2.05±0.21&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.89±0.35&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
<td>1.95±0.18&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>H</td>
<td>30 days 500 mg phenobarbital/kg diet then 30 days NIH-07 control diet</td>
<td>2.66±1.24&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.03±1.03&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND</td>
<td>2.66±0.87&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values represent the mean ± SD of the rate (%) of apoptosis in hepatic focal lesions in dieldrin (10.0 mg/kg) or phenobarbital (500 mg/kg) treated B6C3F1 mice initiated with diethylnitrosamine (two 35 mg/kg injections per week for 8 weeks). ND indicates there was not a sufficient number of focal lesions to evaluate. Statistical significance from treatment Group A<sup>1</sup>, Group D<sup>b</sup>, Group P or Group F<sup>*d</sup> determined by ANOVA (P < 0.05).

Fig. 1. Liver transections through focal lesions induced by diethylnitrosamine (see Materials and methods section) in B6C3F1 mice placed on NIH-07 diet after 30 days treatment with promoting agent (dieldrin or phenobarbital) are shown. Arrows indicate the morphologic presence of apoptosis.

Lesion number and volume in mice fed either dieldrin or PB. Cessation of either dieldrin or PB exposure was accompanied by a decrease in the number and the volume of altered hepatic foci when compared to that seen in mice continuously fed the promoter. The regression of focal lesions following removal of either dieldrin or PB was primarily attributed to the loss of eosinophilic foci. This decrease in focal hepatocytes was directly related to an increased incidence of apoptosis, however, reversion of altered hepatocytes to a phenotype not demonstrable by hematoxylin and eosin staining cannot be precluded. This study indicates that dieldrin and PB promote hepatic focal lesions and that these lesions (eosinophilic in particular) are dependent on continuous exposure to the promoting agent.

Apoptosis has been clearly indicated as a mechanism for elimination of preneoplastic and normal hepatocytes following removal of growth stimulus (44,48–52). Schulte-Hermann and co-workers have shown that N-nitrosomorpholine-initiated focal lesions promoted with PB had drastically elevated incidences of apoptosis after removal of PB treatment (45). An alternative mechanism to explain the loss of focal lesions after promoter withdrawal proposes that focal hepatocytes 'revert' to a phenotype histologically indiscernible from surrounding normal hepatocytes (53). It has been suggested that the actual number of focal lesions do not decrease after promoter cessation, but the rapid loss of volume can be attributed to the loss of detectable phenotype (28). However, the rapid loss of
The relevance of the stability/instability of hepatic lesions to the carcinogenic process is unclear. It has been suggested that the focal lesions that persist after cessation of promoter treatment are the preneoplastic lesions that have the greatest potential to develop into tumors (27-30). Hepatocytes in persistent lesions may have multiple genetic mutations and potential to develop into tumors (27-29). Lesions such as sex steroids, bile acids, and pituitary hormones induce by tumor promoters may occur by persistently enhancing the number of lesions back to control values, the increased apoptosis in focal lesions resulting in the observed decrease number of focal lesions (54).

In the present study, the increased apoptosis in focal lesions occurring after removal of either dieldrin or PB was found to correlate with a decrease in the volume and number of focal lesions. The significance of the stability/instability of hepatic lesions to the carcinogenic process is unclear. It has been suggested that the focal lesions that persist after cessation of promoter treatment are the preneoplastic lesions that have the greatest potential to develop into tumors (27-30). Hepatocytes in persistent lesions may have multiple genetic mutations and may already be in a stage of progression. Conversely, there are numerous endogenous promoting stimuli that have been identified that could enhance the growth of preneoplastic lesions such as sex steroids, bile acids, and pituitary hormones (55-59). In addition, nutritional status e.g. dietary fat intake, tryptophan, methionine or choline deficiency, or sucrose intake may contribute to pathological changes and tumor promotion (55-60). Also, immunological status of the host, species-susceptibility and aging have been shown to contribute to the carcinogenic process (61-64). Since tumor promoting compounds enhance the number and incidence of focal lesions and cessation of promoter treatment results in a decrease in the number of lesions back to control values, the increased incidence of preneoplastic and neoplastic hepatic lesions induced by tumor promoters may occur by persistently enhancing the growth of focal lesions. The chromosome presence of a promoting agent enhances the proliferation and, possibly, inhibits apoptosis, thus increasing the probability of a mutational event required for the step-wise progression to malignancy (65-66).

These studies indicated that preneoplastic lesions promoted by non-genotoxic hepatocarcinogens in mice respond in a similar fashion to rats after removal of promoting stimulus. A variety of studies have shown that some rat hepatic lesions are dependent upon the continuous presence of a promoting agent. This study demonstrates that in mice the growth of some preneoplastic hepatic foci remains promoter-dependent after 30 days of continuous exposure to the promoter. In addition, two hepatic tumor promoting agents studied in this investigation, dieldrin and PB, appear to function by different mechanisms in enhancing focal lesion growth. Further research is required to elucidate the mechanisms behind these differences.

### References


