ABSTRACT

A combination of experimental and simulation approaches were used to analyze the clonal growth of preneoplastic, enzyme-altered foci during liver carcinogenesis in an initiation-promotion regimen. Male Fisher 344 rats, 8 weeks of age, were initiated with a single dose (200 mg/kg, i.p.) of diethylnitrosamine (DEN). Beginning 2 weeks later, animals were exposed to daily gavage consisting of 0.1 mmol/kg pentachlorobenzene (PECB) or hexachlorobenzene (HCB) in corn oil vehicle for 6 weeks. Partial hepatectomy was performed 3 weeks after initiation. Experimental data including liver weight, hepatocyte density (number of hepatocytes/unit volume), S-bromo-2′-deoxyuridine-labeling index for analysis of cell division rate, and number and volume of glutathione-S-transferase-positive foci were collected 23, 26, 28, 47, or 56 days after initiation. Model parameters describing liver growth were obtained directly from the experimental data. The probability of mutation/division of normal cells and the growth rate of initiated cells were inferred by a comparison of model outcomes with the observed time courses of foci development. To describe the time-dependent increases in foci volume and the concomitant reduction of foci number observed in all treatment groups, the calibrated model for the DEN controls incorporated the hypothesis of two initiated cell populations (referred to as A and B cells) within the framework of the two-stage model. The B cells are initiated cells that have a selective growth advantage under conditions that inhibit the growth of A cells and normal hepatocytes. The parameter values defined in the DEN controls were used to evaluate experiments involving the administration of PECB or HCB. Both PECB and HCB caused a significant increase in foci volume compared with the DEN controls. HCB treatments resulted in increased proliferation of normal hepatocytes, which was not observed for PECB under the same treatment regimen. The best description of the data resulted from the model incorporating the hypothesis that PECB and HCB promoted the growth of foci via increased net growth rates of B cells. We present here a biologically based clonal growth simulation platform to describe the growth of preneoplastic foci under experimental manipulations of initiation-promotion studies. This simulation work is an example of quantitative approaches that could be useful for the analysis of other initiation-promotion studies.

INTRODUCTION

Multistage carcinogenesis studies have been extensively used for the analysis of cancer development. In particular, the two-stage initiation-promotion protocol has been widely used in systematic elucidation of the carcinogenesis processes (1). Although the design of these assays varies with respect to the combination and sequence of initiating and promoting events, analysis of putative initiated cells identified by phenotypic markers such as GST-P, continues to be a vital part of studies for understanding the mechanisms of tumor promotion (2–4). The medium-term liver foci bioassay developed by Ito et al. (5, 6) involves the sequential administration of a potent initiator, DEN, followed by chemical treatment and mitogenic stimulation of hepatocyte growth via partial hepatectomy. This protocol allows the evaluation of carcinogenic potential within 8 weeks by identification of GST-P-positive foci as end-point marker lesions. A large number of chemicals have been tested using this protocol. When compared with the 2-year chronic bioassay, results from the Ito medium-term bioassay have correctly identified 97% of genotoxic hepatocarcinogens and 86% of the known nongenotoxic hepatocarcinogens (7).

PECB and HCB are among the 12 chlorobenzene isomers that have been manufactured worldwide for both industrial and domestic uses (8, 9). PECB and HCB are used directly in a few industrial processes and are a waste product or impurity associated with the manufacture of various chlorinated solvents and other registered pesticides (8, 9). Because of the relative persistence of these two chemicals, significant human exposure to both PECB and HCB has occurred (10, 11). The carcinogenicity of HCB has been demonstrated in rats and mice using the 2-year chronic animal bioassay (12, 13). Study of HCB using an initiation-promotion hepatocarcinogenesis protocol also suggests that HCB is a putative liver tumor promoter (3, 13). Whereas the carcinogenic potential of PECB is not known, we have shown previously that both PECB and HCB exposure cause a significant increase in focus formation in the Ito medium-term bioassay (14, 15). Furthermore, both PECB and HCB stimulate production of porphyrin and induce expression of c-fos, c-jun, and cytochromes p450 2E1, 2B1/2, and 1A1 (15).

Statistical and mathematical modeling tools incorporating biological supposition increasingly have been used for examining complex biological problems, including those in neuroscience (16–18) and cancer research (19). Several statistical and computational methods have been used to analyze the experimental data derived from carcinogenesis studies. For example, a biologically based simulation model of carcinogenesis shows that increases in cell proliferation can account for the carcinogenicity of nongenotoxic compounds (19). The stochastic Moolgavkar, Venzon, and Knudson two-stage model of carcinogenesis (20–22) has been shown to be consistent with epidemiological and animal tumor data (20, 21, 23). The two-stage model provides a simplified description of carcinogenic processes with two critical rate-limiting steps: (a) the transition from normal to initiated cells and (b) from initiated cells to malignant states (Fig. 1). Furthermore, relevant biological information such as the kinetics of tissue growth and differentiation and mutation rates may also be incorporated. Both exact and approximate analytical solutions have been derived for the two-stage model (24–26). Recently, these quantitative
models have also been used to analyze the growth of preneoplastic enzyme-altered foci data, including studies using TCDD, polychlorinated biphenyls, phenobarbital, and α-hexachlorocyclohexane (27–31). The results of these analyses have led to interesting conclusions and have indicated specific studies to verify model predictions.

A numerical simulation model for the clonal growth of foci was described independently by several investigators (31–33). Although based on biological parameters similar to the Moolgavkar, Venzon and Knudson model (20–22), the numerical model uses a different mathematical construction. To represent the multiplicity of the cellular states and the time-varying nature of the numerous cell behavior variables, the numerical model resorts to a recursive simulation. The growth of normal liver, in the work by Conolly et al. (31, 33), is described deterministically, whereas other cellular events use stochastic simulation. Model output can be compared with experimental data including target organ size (total number of cells), cellular proliferation, and the observed time course of foci development. This approach facilitates description of complex biological processes with time-dependent values.

Despite the large data set generated from the Ito medium-term bioassay (7), few quantitative analyses of these studies have been attempted (32). To analyze the time-course preneoplastic foci data from the Ito medium-term bioassay collected in our laboratory, we have used the clonal growth simulation model of Conolly et al. (31, 33) previously. We have demonstrated a successful simulation of foci growth kinetics for PECB (34). In this study, the clonal growth modeling platform for the Ito medium-term bioassay was improved by incorporating time-dependent changes in hepatocyte density, hepatocyte proliferation, and a description of foci growth kinetics in the DEN control group. The goal of the current study was twofold. First, time-dependent relevant biological information of PECB or HCB–induced foci development was collected to calibrate parameters of the clonal growth model in the context of the Ito medium-term bioassay. Second, the utility of the clonal growth model as a hypothesis-testing tool was evaluated to derive quantitative biological information regarding potential mechanisms of carcinogenesis for PECB and HCB.

MATERIALS AND METHODS

The method used in the present study consists of two main parts: experimentation and simulation modeling. Experimental data collection included time course measurements of liver growth, hepatocyte proliferation, and foci development. These data were then used for subsequent clonal growth simulation modeling.

Experimental Data Collection

Chemicals

PECB and HCB were purchased from Aldrich Chemical (Milwaukee, WI). DEN was purchased from Sigma Chemical Co. (St. Louis, MO).

Animals and Treatment

Male Fisher 344 (F344) rats, 30 days of age, from Harlan Sprague Dawley (Indianapolis, IN) were acclimated for 4 weeks before the start of the experiment. The rats were randomized by weight and divided into three treatment groups (Fig. 2). Male rats received a single i.p. injection of DEN (200 mg/kg) dissolved in 0.9% saline. After 2 weeks, the rats received daily gavage administration of corn oil or 0.1 mmol/kg PECB or HCB in a corn oil vehicle through the remainder of the 8-week study. At week 3 (day 21), a partial hepatectomy was performed on all animals. To examine the consequence of partial hepatectomy, five animals from each treatment group did not receive partial hepatectomy and were killed on day 23. Concurrent controls without DEN initiation were also included for the three treatment regimens. Animals were given food (Harlan Teklad NIH-07 Diet; Madison, WI) and water ad libitum, and lighting was set on a 12-h light/dark cycle. On days 23, 26, 28, 47 and 56, at least five animals from each treatment group were killed by aortic exsanguination (Fig. 2). Whole livers were removed; tissues were fixed in either 10% neutral-buffered formalin or ice-cold acetone, embedded in paraffin, and serially sectioned at 5 μm. The studies were conducted in accordance with the NIH guidelines for the care and use of laboratory animals. Animals were housed in a fully accredited American Association for Accreditation of Laboratory Animal Care facility.

GST-P Foci

Acetone-fixed tissues were used for the immunohistochemical identification of GST-P foci (14). Liver sections were paraffin embedded and rehydrated by passage through an alcohol series. Endogenous peroxidase was quenched in 3% hydrogen peroxide for 10 min. The slides were rinsed with deionized water and placed in PBS (pH 7.4; 2.7 mM KCl, 0.14 M NaCl, 1.5 mM KH 2 PO 4 , and 8.1 mM Na 2 PO 4 ). A standard avidin-biotin complex method protocol (Vector Labs, Burlingame, CA) was followed, and foci were detected with GST-P primary antibody (Binding Site, San Diego, CA). GST-P foci were visualized using the ABC method (Vector Labs, Burlingame, CA) and diaminobenzidine (DAB) as the chromogen.
measured using a Leitz light microscope coupled with the BioQuant image analysis system (version 5; R&M Biometrics, Nashville, TN). Foci bigger than two cells, roughly corresponding to 50 μm in diameter, were recorded.

**Determination of Cell Division Rate**

Osmotic minipumps (Alzet model 2 ML1, 10 μl/hr; Alza Corporation, Palo Alto, CA), filled with BrdUrd (20 mg/ml), were implanted s.c. over the dorsal midscapular region. Animals were anesthetized with isoflurane (Anaquest, Madison, WI), and the incision was closed using stainless steel wound clips. To avoid saturation of labeling, pumps were implanted 1 day before tissue collection on time points soon after partial hepatectomy (days 23, 26, 28). For other time point collections, pumps were implanted 3 days before the animals were killed. Control animals not hepatotoxicimated were given BrdUrd for 3 days. Detection of BrdUrd-labeled cells was performed on formalin-fixed liver sections using standard avidin/biotin complex method immunoperoxidase kits (Vector Labs, Burlingame, CA) with primary BrdUrd antibody (Biogenex Labs, San Ramon, CA) and 3-amino-9-ethylcarbozole (Biomedex, Foster City, CA). At least 1000 cells/animal and four animals/group were counted. The labeling index (LI) was calculated as the number of cells labeled divided by the total number of cells counted. The cell division rate (α, day⁻¹) was calculated as described by Moolgavkar and Luebeck (35):

$$\alpha = \frac{1}{2t} \times \ln \left( \frac{1}{1 - LI} \right)$$

where t is the number of days of exposure to BrdUrd.

**Cell Morphometric Analysis**

Liver sections fixed in formalin were stained with H&E to evaluate liver architecture and cell morphology. A point-count-intersect method was used to determine volume density and to quantify nuclear and cytoplasmic areas (36). Ten random fields/section and at least four animals/group were analyzed. The relative ratios/unit area (i.e., area density) were determined by categorizing intersect points as hepatocyte nucleus, hepatocyte cytoplasm, or others (sinusoid or erythrocyte). The numerical density of hepatocytes was estimated from counts of hepatocytes in a test area of known size and divided by hepatocyte volume density using the formula described by Weibel et al. (36).

**Stereology Methods**

Quantitative stereology data for each rat were obtained by using STEREO, a Windows 98/NT program developed at the McArdle Laboratory, University of Wisconsin, Madison, WI (37). STEREO uses a data file containing tissue information (tissue areas and individual focal transections) as the input to provide quantitative stereology results on a three-dimensional basis. Numbers of foci/cm³ of liver were calculated according to the method of Saltkyov (38). The Saltykov size classes were defined on the basis of a logarithmic scale of diameters with the factor $10^{0.1}$ (the ratio of maximum diameter of two neighbor classes is equal to $10^{0.1}$; 0.7943). Calculations were performed at the truncation value of 50.12 μm. Preneoplastic foci are classified according to Saltkyov size classes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11, with the maximal diameters of 63, 79, 100, 125, 154, 199, 251, 316, 398, 501, and 630 μm in each class (37, 38). GST-P foci size distributions were studied on the basis of group-combined data. In this case, data files from the same group were combined first on a two-dimensional level, and then three-dimensional data (the number of the foci in each size class) were calculated according to the method of Saltkyov (37, 38). The volume fraction of the liver occupied by GST-P foci was computed by the method of Delesse (39).

**Clonal Growth Simulation Modeling**

**Description of the Clonal Growth Model**

The simulation model used for the current analysis was based on the clonal growth model described previously (31, 33). Part of the simulation algorithm was modified to reflect different times of administration of DEN initiator, promoters, and partial hepatectomy. A summary of the basic modeling framework is provided here.

(a) Normal Cell Growth. Growth kinetics of the hepatocyte are described as a function of division (α; 1/day) and death rates (β; 1/day) deterministically by

$$\frac{dN}{dt} = N(\alpha - \beta)$$

where N is the number of normal hepatocytes/cm² and β represents all modes of cell death including apoptosis and necrosis.

(b) Mutation to Initiated Cells. The expected number of initiated cells generated is modeled stochastically and is linked to the number of hepatocyte cell divisions during each time step $\Delta t$ by

$$N_m = N_\mu \alpha \Delta t$$

where $N_m$ is the number of normal hepatocytes mutating during the time interval $\Delta t$, and $\mu$ is the probability of mutation/cell division. A random deviate about $N_m$ denoting the number of mutations during $\Delta t$ is drawn from a Poisson distribution using the function PODEV (40). Inputs to PODEV are the mean of the Poisson distribution and a pseudorandom number between 0 and 1 generated with the algorithm UNFL (41). Our initial effort described the first stage of the two-stage model (from normal cells to initiated cells) for the GST-P foci data (Fig. 1). The possibility of two initiated populations for adequately describing the kinetics of foci growth was also evaluated. In this case, the probability of mutation/cell division to A or B initiated cells are denoted by $\mu_A$ and $\mu_B$. The division and death rates of A and B cells are denoted by $\alpha_A$, $\beta_A$, $\alpha_B$, and $\beta_B$, respectively (Fig. 1).

(c) Growth of Initiated Cells. The growth of initiated cells is modeled with a random event in which initiated cells can undergo cell division, or cell death, or no change in a given time step. The division of single mutated cells derived directly from normal cells may give rise to clones of initiated cells (preneoplastic foci). The program keeps track of each clone over time, allowing average clone size (cells/clone) and number (clones/cm³) to be described. The simulation results can then be compared with three-dimensional foci volume fraction (calculated from average clone size and number of clones/cm³) and foci number data converted from two-dimensional focal transection data by the stereological methods as described above.

(d) Other Model Details. Throughout the simulation, the model keeps track of the total hepatocyte number in the liver. Simulated liver weight is calculated on the basis of the total hepatocyte number divided by the corresponding hepatocyte density (number of hepatocytes/unit volume). Partial hepatectomy is described as an instantaneous decrease in liver weight and cell number. As observed in our experiments, the remaining liver began regeneration 24 h later and was fully regenerated in 7–10 days. In the simulation model, regeneration is achieved by increasing the hepatocyte division rates ($\alpha$). The enlargement of the liver through hypertrophy, a common response after exposure to many tumor promoters (42), was incorporated by including a time-dependent change in hepatocyte density. For comparing the simulation output with the foci data collected, only the clones larger than two cells in size were considered detectable. Growth of clones larger than 1000 cells was described deterministically (Eq. B). This simplification speeds computation and provides results equivalent to a fully stochastic calculation (33). We confirmed that the simulations were equivalent for the two different modeling strategies with the parameter sets used in this paper (results not shown).

**Modeling Strategies**

Analysis of several studies indicated that after a sufficiently large dose of DEN, the cell division rate of initiated hepatocytes decreases with time (43), whereas the death rate of initiated cells increases with time (44). Although detailed kinetic data are currently unavailable to define a continuous function describing this time-dependent variation in growth rate, the strategy for implementing the time-dependent changes in the current model is via the use of piecewise constant parameters.

(a) Time Intervals for Piecewise Constants. The modeling time frame was divided into six time intervals with constant parameters in each interval. The choice of such intervals was partly based on a review of the literature on...
the time course of foci formation after single-dose of DEN treatment. GST-P-positive foci were detectable 4–5 days after a single dose of DEN (200 mg/kg). The first significant increase in the total number of GST-P foci was observed after 7 days, with the maximum observed on day 14. Total foci numbers then dropped to a minimum after 28 days, and again increased to higher levels by day 120 (45, 46). This pattern of DEN-induced foci kinetics was also noted in the study by Jang et al. (47) that included a detailed description of individual foci growth categorized by their size at different time points after a single dosing of DEN (200 mg/kg). We transformed these data (47) by converting the number of hepatocytes recorded in the foci to the size of foci using the assumption that one hepatocyte roughly corresponded to a transection diameter of 25 μm (48). These foci-size distribution data were then converted to three-dimensional foci volume and number using the stereological methods, as described above. Two time intervals (days 1–7 and days 7–14) were chosen to reflect the first high elevation of GST foci number on day 7 (47) and administration of potential promoters on day 14 in the Ito assay. The time interval from day 21 to day 28 was included because of the effects of partial hepatectomy on cell kinetics. The study by Satoh et al. (45) described DEN-induced changes in the number of GST-P-positive foci after partial hepatectomy from day 28 to day 120, with the lowest foci number observed around day 56. To ensure that the foci volume and foci number of modeling outputs would approximate the results observed by Satoh et al. (45), two additional time intervals, day 28 to 35 and day 35 to 120, were introduced.

(b) Obtaining Values for Model Parameters. Once the six time intervals (days 1–7, 7–14, 14–21, 21–28, 28–35, and 35–120) were chosen, parameter values associated with the growth of liver (normal hepatocyte division/death rates, α and β; Fig. 1) were obtained from the experimental data collected in the current time course study and from published literature. The mutation rate (μα and μβ; Fig. 1) and division/death rates of initiated cells (α0, β0, αi, and βi; Fig. 1) in all treatment groups were estimated in a stepwise fashion as described below.

Model Calibration for Hepatocyte Growth Parameters

This calibration exercise estimated the values of normal hepatocyte cell division and death rates for the Ito protocol. The values estimated are species- and age-dependent, but should not vary significantly from experiment to experiment. Proper parameterization of liver growth parameters was a basis for estimation of the mutation rates and division/death rates of initiated cells because the mutation rate is a function of normal hepatocyte division rates (Eq. C). Normal hepatocyte division rates at the various time points were estimated from experimental measurements. In addition to the current investigation, data from studies characterizing the cell division and cell death kinetic profile after a single dosing of 200 mg/kg DEN (49) were used to calibrate the cell birth/death rate and growth of liver in the DEN control group. The hepatocyte basal cell division rate was set to 0.0015 day⁻¹, the lowest rate from our time course measurements of BrdUrd-labeled cells. Hepatocyte regenerative proliferation as a result of DEN cytotoxicity was characterized on the basis of a published study (49) with an exponential function. The hepatocyte cell division rates from day 14 to day 120 were based on BrdUrd-labeling data collected in the present study. Changes in cell division rates after partial hepatectomy were based on BrdUrd-labeling data and fitted using a decreasing exponential function. Because there was no significant growth of normal liver during the course of study, the basal death rate was set to be the basal birth rate, 0.0015 day⁻¹. The death rate of normal hepatocytes during the period after DEN treatment was derived using the data published by Kato et al. (49). Once the growth kinetics of normal hepatocytes (division and death rates) were defined, these rate constants, along with time-dependent changes in hepatocyte density, were used to simulate changes in liver weight and were compared with the experimental data collected. At the beginning of the study, the liver was treated as consisting of 1.69 × 10⁶ hepatocytes/cm³ (36).

Estimation of Mutation Rates

Parameterization of mutation rates for the different time intervals was based on the combination of literature support and fitting of our experimental data. The work by Dragan et al. (50) measured the concentration of three different types of DNA adducts after a single dosing of DEN. On average, the concentration of these adducts remained significantly elevated during the first 7 days and returned to basal levels after 2 weeks. Assuming that the mutation rate was positively correlated with the concentration of DNA adducts, we allowed the mutation rates to vary in three different time intervals with the first 7 days having the highest mutation rates (i.e., days 1–7; Table 3). With this qualitative information, the probability of mutation/cell division was subsequently estimated on the basis of the total number of foci observed. Thus, the highest number of detectable foci in any of the treatment groups (DEN without partial hepatectomy, DEN controls with partial hepatectomy, DEN+PECB, and DEN+HCB groups with partial hepatectomy) was used as an approximation of the number of mutated cells available for promoting action.

Estimation of Growth Rates of Initiated Cells: Model Calibration for the DEN Controls without Partial Hepatectomy

The time course labeling index in the initiated cells (hepatocytes contained within foci) was not determined in the current work. Previous modeling exercises indicated differential growth characteristics among initiated cells (31, 34). The growth parameters associated with 2 types of initiated cells were thus estimated on the basis of their correspondence with time course foci data. The work by Jang et al. (47) described the time course changes in both foci number and percentage of liver volume in animals receiving DEN treatment but without partial hepatectomy. The division and death rates of initiated cells before partial hepatectomy were therefore inferred by a comparison of model outcomes with the data converted from Jang et al. (47).
Table 2 Determination of cell proliferation rate of normal hepatocytes in liver tissues of F344 rats subjected to an initiation/promotion protocol using DEN as an initiator and PECB or HCB as promoting chemicals

| Time Course Changes in Cell Division Rates. | Time-dependent and treatment-dependent changes in liver weight, cell proliferation, foci number, and hepatocyte density were analyzed using a mixed-effects ANOVA model, and then by post-hoc Tukey-Kramer multiple comparison tests at \( P = 0.05 \). The analysis was programmed using the SAS system (SAS Institute, Inc. Cary, NC).

RESULTS

Experimental Data Collection

Time Course Changes in Body and Liver Weights. The effects of PECB or HCB on final body weights and liver weights are summarized in Table 1. Exposure to HCB, 0.1 mmol/kg/day for 6 weeks, led to a significant increase in (relative) liver weight at the end of the study compared with the DEN control rats (\( P < 0.05 \)). Exposure to PECB at the same dose and duration did not cause a significant increase in (relative) liver weight, although the trend was observed. Treatment with PECB or HCB alone without DEN initiation resulted in liver weight changes similar to those of the DEN+PECB and DEN+HCB groups. The final liver weights (expressed as a percentage of the total body weight) in groups receiving only PECB and HCB are 4.1% and 6.7%, respectively. The increased liver weight in the DEN+PECB and DEN+HCB groups was especially pronounced after partial hepatectomy (Table 1). Neither PECB nor HCB had caused a significant deviation in total body weight at the end of the 8-week study (Table 1).

General Pathology and Morphometric Analysis. Histopathological examination of H&E-stained liver sections of PECB or HCB-treated animals showed hepatocellular hypertrophy in the centrilobular region. Our initial modeling exercise predicted a marked reduction of hepatocyte density (number of hepatocytes/unit volume) in the DEN+HCB group. To quantify hepatocyte density as necessary for a good description of the model to the data, we used a quantitative morphometric analysis. Hepatocyte density in the DEN+PECB and DEN+HCB groups gradually declined, with the most significant reduction observed on days 47 and 56 (\( P < 0.05 \); Fig. 3). This reduction was much more pronounced in the DEN+HCB group than in the DEN+PECB group (Fig. 3). The reduced density was attributable to increases in both cytoplasmic and nucleus volumes, as previously reported (14).

Time Course Changes in Cell Division Rates. Time-dependent changes in cell division rates of normal hepatocytes were determined quantitatively stereology results on a three-dimensional basis is available from XU.\(^5\)

Statistical Analyses

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by the BrdUrd-labeling index among DEN controls, DEN+PECB, and DEN+HCB groups (Table 2). The basal division rate of normal hepatocytes was approximately 0.0015 day\(^{-1}\) (Table 2). As shown in Table 2, there were ~33- to 58-fold increases in the division rate of normal hepatocytes subsequent to partial hepatectomy in all treatment groups. On day 28, except for the DEN+HCB group, the cell division rate had returned to approximately within 1- to 2-fold of normal basal levels. PECB treatments did not cause any significant changes in cell division rates compared with DEN controls at any of the time points examined. However, HCB treatments resulted in significant increases in cell division rates relative to the DEN control at later treatment time points (days 28 and 47). The means of the cell division rate in the DEN+HCB group were between 124% to 376% of concurrent DEN controls.

**Time Course Changes in Foci Number, Volume, and Size Distribution Profile.** As seen in Fig. 4, foci volume increased from day 23 to day 56 for all three groups. Foci number initially increased during the week after partial hepatectomy, with a significant decline thereafter. Both PECB and HCB caused a significant increase in foci volume at later time points (days 47 and 56) compared with the DEN control group (\(P < 0.05\)). Thus, time-dependent reduction in foci number was accompanied by a concurrent increase in foci volume in all treatment groups. The various sizes of preneoplastic foci from day 23 to day 56 were classified according to the method of Saltykov (38). At an early time point (day 23), the initiated population consisted of mostly small foci (Fig. 5). On day 56, the proportion of small foci was significantly lower for all three groups. Analysis of time-dependent changes in foci size profiles indicated that there was a slow accumulation in the number of foci for all size classes in the DEN+PECB group when compared with the DEN control group (Fig. 5). For the DEN+HCB group, there was a slow loss of small size class foci (i.e., the number of foci in size classes 1–4) over time. By day 56, there were hardly any small foci present in the DEN+HCB group.

**Clonal Growth Simulation Modeling**

**Model Calibration for Hepatocyte Growth Parameters.** A peak increase in cell death rate occurred within 6 days after DEN administration. The increased cell death rates accounted for the decreased liver weights observed shortly after DEN treatment (49). In addition, after necrotic cell death, there was a regenerative proliferation response (49). The model incorporating these data shows a drop in liver weights (Fig. 6A) and a peak increase in cell division rates immediately after DEN treatment (Fig. 6A). To reflect the experimental findings (Table 2), a 5-fold increase above basal cell division rates after DEN treatment was incorporated in all treatment groups up to day 21. Partial hepatectomy resulted in another increase in cell division rates (Fig. 6A). A 2.5-fold increase of basal cell division rates was incorporated in modeling the DEN+HCB group from day 28 to the end of the modeling period (Fig. 6A). With calibrated cell division rates and hepatocyte density, the model described the time-dependent changes in liver weights in all three groups (Fig. 6B). Model exercises using the measured hepatocyte density data indicated that hypertrophy, resulting in a decreased hepatocyte density, accounted for the increased liver weights in the DEN+HCB group. Thus, the increased liver weights in the DEN+HCB group were attributable to hypertrophy and not to the increased cell number (Fig. 6B).

**Model Calibration for the DEN Controls without Partial Hepatectomy.** The parameterized model adequately describes the foci growth data of Jang et al. (47), in which time course data were collected after a single dose of 200 mg/kg DEN without partial hepatectomy (Fig. 7). For example, the model describes the peak formation of foci volume and number 14 days after DEN administration, with a decline thereafter (Fig. 7). It also successfully describes an increase in foci volume around 60–80 days after the initial decline.
subsequent to DEN dosing (Fig. 7B), an observation that has been reported by several other investigators (45, 46).

Model Calibration for the DEN Controls. Two working hypotheses were tested regarding the nature of initiated cells formed resulting from DEN treatment. As shown in Fig. 4, the reduction of the total foci number at later time points was accompanied by an increase in foci volume in the DEN controls. Decreased total foci number indicated that some of the foci were eliminated via cell death. The observed high foci volume fraction with decreased total foci number indicated that some of the foci underwent significant growth. To reproduce the shape of these time course changes, the modeling exercise based on the hypothesis of one initiated cell population did not yield satisfactory results. The best simulation of the DEN time course data were obtained with two initiated populations (A and B cells) with distinct growth characteristics (i.e., cell division and death rates). The B cells were assumed to have a selective growth advantage under conditions that inhibit the growth of A cells and normal hepatocytes. The final model adequately describes the foci data of the DEN controls (Fig. 7). Interestingly, the model suggests that partial hepatectomy creates rapid increases in foci number and volume (Fig. 7) that facilitate the detection of foci within a short time frame. For example, the foci volume with partial hepatectomy is ~7-fold higher than the one without partial hepatectomy at day 30 (Fig. 7). The parameters used for the simulation under the hypothesis of two initiated cell populations are summarized in Table 3. The mutation rate to A cells is 0.002–0.006 day$^{-1}$. The growth characteristics (death or division rates) of A and B cells do not differ significantly from each other before the administration of promoters on day 14. For both A and B cells, it appears that cell death mechanisms are activated in response to the excess growth of initiated cells (i.e., the death rates of A and B cells are high from days 28–35 and days 35–120; Table 3). The death rate of A cells tends to be higher than that for B cells. The death rate of A cells is 0.08 day$^{-1}$ on days 14–21, compared with 0.002 day$^{-1}$ for B cells at the same time interval. Partial hepatectomy appears not only to accelerate the cell divisions of A and B cells but also to inhibit the death of both A and B cells (days 21–28; Table 3).

Modeling Analysis of DEN+PECB and DEN+HCB Data. An iterative fitting to the time course foci volume and number data eventually led to a set of parameters, indicating that both PECB and HCB increased the cell proliferation of initiated cells. The differences between model predictions and foci kinetic data of PECB or HCB were significantly larger when assuming PECB and HCB as mutagens. The modeling outputs generally fall in the range of experimental data (Figs. 8 and 9). Whereas PECB or HCB was administered starting on day 14, foci growth patterns resulting from these treatments did not differ significantly from those of the DEN control group until partial hepatectomy was applied (Figs. 8 and 9). PECB and HCB cause an additional boost in the proliferation of A cells immediately after partial hepatectomy (days 21–28) when compared with the DEN controls (Table 3). The cell division rate of A cells during days 21–28 in the DEN+PECB group is 1.5-fold that in the DEN controls. The model describes a rapid increase in foci volume as a result of PECB or HCB treatments during the 2 weeks after partial hepatectomy. These rapid increases reach a plateau around day 35, regardless of
continuous treatments of PECB or HCB (Figs. 8B and 9B). PECB results in a larger increase in the number of foci after partial hepatectomy compared with DEN controls (Fig. 8A). This increase in foci number is then followed by a rapid decline (Fig. 8A). Negative selection hypothesis of tumor promotion (52, 53) was successfully used to describe time course foci development in the DEN+PECB and DEN+HCB treatment groups (Table 3). PECB and HCB each could increase the net growth rate of B cells via the reduction of B cell death rates. For example, when minimal biologically plausible death values of B cells from days 28–35 (0.05 day⁻¹) in the DEN controls was assumed (Table 3), the death rate of B cells is 0.04-fold that of DEN controls from day 28–35 in the DEN+PECB group, and the death rate of B cells is 0.22-fold that of DEN controls from day 28–35 in the DEN+HCB group. PECB also appears to inhibit the death of A cells (Table 3). A result of diminished cell death is an increase in the number of foci, for both large and small foci, compared with the DEN group, a prediction that is consistent with our experimental analysis for the DEN+PECB group (Fig. 5). The model suggests that exposure to HCB after DEN initiation gradually creates an environment that is growth-inhibitory to A cells, with B cells gaining a growth advantage. Whereas HCB promotes the growth of B cells, it accelerates the death of A cells at the same time (i.e., the death rate of A cells is 1.5-fold that of DEN controls from days 28–120; Table 3), resulting in a profile of foci size distribution predominated by large foci. This modeling output reproduces the size distribution profile observed for animals in the DEN+HCB group. The comparison of modeling outputs with and without the incorporation of time-dependent hepatocyte density changes indicates that the incorporation of density data significantly improves the description of the model to the data (Fig. 9A).

Fig. 8. Comparison of the clonal model outputs with experimental measurements of foci growth in the DEN+PECB group. The data are expressed as mean ± SD of at least four animals at each time point. Results from five simulation runs are shown. The simulation for DEN controls (DEN) is plotted to illustrate the relative potency of PECB in inducing foci growth.
DISCUSSION

We demonstrate here the application of a clonal simulation model for describing PECB- or HCB-induced time-dependent changes in foci growth within the Ito medium-term bioassay. The parameterized model also adequately describes DEN-induced foci growth data published previously (47). On the basis of the framework of the two-stage model (22), the stochastic simulation model used for this work illustrates an easy incorporation of time-dependent biological parameters such as normal hepatocyte division/death rates, liver weight, and hepatocyte density. Consistent with previous simulation results for PECB (34), the current modeling exercise indicates that the use of two initiated populations (called A and B cells) yields the best simulation of time course data for both PECB and HCB studies. The modeling outputs also suggest a potential mechanism for tumor promotion of PECB and HCB by increasing the net growth rate of B cells, a population of initiated cells that appear to escape the inhibitory homeostatic environment (52, 53) after exposure to PECB and HCB.

Negative Selection Hypothesis of Tumor Promotion. The combination of experimental observations and clonal growth modeling within the framework of the two-stage assumption strongly indicates that there are two distinct types of initiated cell populations that differ in their responses to the negative regulatory/homeostatic mechanisms. These results lend additional support to the negative selection hypothesis regarding the biological mechanisms of several tumor promoters (52). The negative selection mechanism for hepatic tumor promotion has been proposed and characterized for phenobarbital (53). Exposure to liver promoters, such as phenobarbital, appears to transiently enhance hepatocyte proliferation, leading to a homeostatic increase of mitoinhibitory growth factors, such as TGF-β1, to constrain proliferation (53, 54). Under this negative selection environment, the growth of a second population of cells with mutations rendering them insensitive to the compensatory inhibitory responses is possible (53, 54). This population, referred to as B cells in the present study, appears to have growth advantages in the presence of persistent mitosuppression. Experimental works supporting the negative selection mechanism include studies demonstrating that the mature form of TGF-β1 is involved in the initiation of apoptosis in the liver (55) and that pericentral-equivalent hepatocytes are more sensitive to TGF-β1-induced apoptosis than periportal-equivalent hepatocytes (56). Loss or alteration of function of the mannose-6-phosphate/insulin-like growth factor II receptor, which is required for TGF-β1 activation, may account for the escape of these cells from TGF mitosuppression (57, 58). Furthermore, down-regulation of TGF-β receptors is observed in phenobarbital-promoted hepatic tumors (59), indicating that reducing the ability of TGF-β to inhibit cell growth may confer a selective growth advantage to the initiated cells. To validate the current modeling outputs, additional experimental work is needed to identify the growth regulatory factors, such as TGF-β1, that might be involved in a negative selection mechanism for HCB and PECB.

Although DEN treatment may well result in more than two types of mutated cells, the modeling suggests that for the purpose of describing focus growth, the cells can be lumped into two types, A and B cells. In fact, experimental work on this assumption has been provided. Dragan et al. (50) characterized three promutagenic lesions after DEN treatment and found that two O-alkylpyrimidine adduct groups have a significantly longer half-life than does the other adduct group. This biphasic DNA adduct kinetics is correlated with the biphasic kinetics of GST-positive foci and BrdUrd-labeling index (50), supporting our two-cell structure of the model.

Using the clonal simulation model as a hypothesis-testing tool, we...
showed that both PECB and HCB are likely to affect the cell proliferation of initiated cells, but not as direct-acting mutagens. PECB or HCB exposure after DEN initiation resulted in time-dependent increases in foci volume that were significantly larger than those for the DEN controls. We were able to construct a model to adequately describe the time course data sets of PECB and HCB based on the negative selection hypothesis. In this model, PECB and HCB treatments resulted in the growth of B initiated cells with reduced sensitivity to compensatory inhibitory responses (i.e., reduced cell death rates). Effects on the cell death rates of initiated cells have been seen with other tumor promoters, including TCDD and phenobarbital, from both experimental data and model predictions (4, 60). However, the available experimental data could not allow the model to rule out the possibility that PECB and HCB could affect the division rate of B initiated cells. Modeling exercises based on the hypotheses that PECB and HCB each affect either the cell division or cell death rates of B cells led to equal descriptions of the experimental data, indicating that the net growth rate (division rate – death rate) of B cells is what determines the growth patterns of foci. On the other hand, the differences between the effects of PECB and HCB on the cell kinetics of normal hepatocytes and their marked differences in foci size distribution profiles indicate possible differential mechanisms of PECB and HCB in altering foci growth. Most notably, the model predicts that PECB could strongly inhibit the death rate of B cells, as low as 4% of values estimated for DEN controls. This prediction would not have been possible without any type of quantitative analysis. Although the exercise presented here does not, of course, prove that this is the mechanism of action for PECB, it indicates that it is analytically possible. Thus, the clonal growth model provides a quantitative platform for comparing promotion potency and mechanistic hypotheses for carcinogenesis within the framework of the two-stage model.

Calibration of Model for a Medium-term Liver Bioassay. The significance of the current work includes an initial parameterization of the clonal growth model for the analysis of preneoplastic foci growth for the Ito medium-term assay, which has been used to characterize a large number of chemicals (5, 6). The clonal growth model can be viewed as an assembly of dynamic relationships between variables that contribute to preneoplastic foci formation and growth. Several important kinetic relationships are established in the present study, including a description of the quantitative consequences of partial hepatectomy and high-dose DEN treatment in inducing foci growth and altering normal cell growth. Partial hepatectomy appears not only to accelerate the divisions of A and B cells, but also to trigger a temporary blockade of apoptosis of both A and B initiated cells. According to our model prediction, partial hepatectomy on days immediately subsequent to day 14 would result in a relatively high foci volume by significantly blocking the deaths of A and B cells (Fig. 7). The effects of the timing of partial hepatectomy on the induction of preneoplastic liver foci were investigated to determine the most effective schedule for the medium-term bioassay system (45). The present model substantiates the original design of the Ito medium-term bioassay, in which the time of partial hepatectomy on day 21 promotes the growth of foci more effectively than on day 28 or later (45). The current modeling also reproduces previously observed patterns in the induction of GST-P hepatocytes by DEN, including peak growth at 14–16 days after initial DEN treatment (49) and a rebound in growth around day 60 after initial decline (45, 47). Knowledge of the quantitative consequences of partial hepatectomy and DEN treatment will be important for the comparison and interpretation of the results of the medium-term bioassay with those from the 2-year chronic bioassay.

Experiments to Verify the Current Model Hypothesis. An important strength of simulation modeling lies in its ability to predict and reveal critical biological properties that may be verified by additional experimentation. Our simulation results indicate that the mechanism of tumor promotion exerted by PECB or HCB is via the increased net growth rate of B cells, the initiated cell population with a selective growth advantage under conditions that are inhibitory to the other type of initiated cells (A cells) and normal hepatocytes. The inclusion of this two-cell hypothesis has also been used for a successful description of TCDD-induced foci data (31). To validate this prediction, identification of phenotypic markers and molecular events associated with foci formation and growth of the A and B cells will be valuable. Future experimental work should also include concurrent determination of the cell proliferation rate and cell death/apoptosis kinetics within A or B foci identified by specific phenotypic markers. In addition, we found that the magnitude of parameter changes in the DEN+PECB and DEB+HCB groups compared with the DEN controls were much larger when assuming that PECB and HCB each affect the cell death rate of B cells instead of the cell division rate (PECB from day 28–35: 0.04-fold of the DEN controls assuming the effects on the cell death versus 1.4-fold of the DEN controls assuming the effects on the cell division; HCB from day 28–35: 0.22-fold of DEN controls assuming the effects on the cell death versus 1.3-fold of the DEN controls assuming the effects on the cell division). These predictions will help future experimental design and need to be experimentally verified.

Whereas the current work simulated the first stage of a two-stage model, a similar approach can be used to complete the second stage of the two-stage model. Using available dose-dependent and time course foci and tumor data, such as those from the 2-acetylaminofluorene study (46, 61), this work could be extended to improve our understanding of the shape of the dose-response curve for preneoplastic foci induction and, ultimately, for tumor formation. The utility of the clonal growth simulation model to facilitate the design of a more effective chemoprevention protocol in a carcinogenesis model may also be possible (62). As initiation-promotion protocols are widely used in the study of carcinogenesis, the clonal simulation of foci growth will be a useful quantitative tool for examining dynamic changes at cellular levels during carcinogenesis.

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A Clonal Growth Model: Time-course Simulations of Liver Foci Growth following Penta- or Hexachlorobenzene Treatment in a Medium-term Bioassay

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