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Reduced DNA adduct formation in replicating liver cells during continuous feeding of a chemical carcinogen

(chemical carcinogenesis/immunohistochemistry/carcinogen–DNA modification/acetylaminofluorene/DNA replication)

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ABSTRACT **To investigate early cellular alterations in liver DNA during hepatocarcinogenesis, we have visualized replicating cells and analyzed their DNA adduct content in livers of rats continuously fed a carcinogenic level (0.02%) of 2-acetylaminofluorene for periods up to 4 weeks. One hour prior to sacrifice, cells undergoing DNA synthesis were pulse-labeled with the thymidine analogue 5-bromodeoxyuridine. Replicating cells were visualized immunohistochemically with anti-(5-bromodeoxyuridine), and identification of aminofluorene–DNA adducts in replicating nuclei was achieved by staining with an antiserum specific for *N*-(deoxyguanosin-8-yl)-2-aminofluorene; both stains were observed simultaneously by two-color immunofluorescence. Data were obtained for all cells, including large hepatocytes (nuclei > 6 μm) and small cells (nuclei < 6 μm), such as hepatocytes sliced asymmetrically, oval cells, Kuppfer cells, and sinusoidal lining cells. Based on the size of their nuclei, the hepatocytes were the only cells that could be identified separately from the total. A distinct increase in the number of cells synthesizing DNA was observed after 25 days of 2-acetylaminofluorene feeding; replicating cells were either scattered randomly throughout the liver or clustered in discrete foci. At times up to 28 days, cells with both large and small nuclei that were synthesizing DNA showed reduced aminofluorene–DNA adduct immunofluorescence compared to nonreplicating cells. The results suggest that liver cells replicating during carcinogen exposure have altered metabolic capacities resulting in reduced aminofluorene–DNA adduct formation. It is possible that such cells constitute the progenitors of preneoplastic foci, which have a replicative advantage as compared to normal liver.**

In previous studies, accumulation of the DNA adduct *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) was demonstrated in rat liver nuclei during 1 month of continuous feeding of 0.02% 2-acetylaminofluorene (AAF), prior to the induction of enzyme-altered foci (1, 2). Later, depleted dG-C8-AF adduct formation was demonstrated in all identified enzyme-altered foci induced by four different hepatocarcinogenesis protocols and challenged with AAF feeding for several days immediately prior to sacrifice (3). Enzyme-altered foci are preneoplastic liver lesions known for decreased metabolic activation, increased detoxification and scavenger pathways (4–7), and resistance to the effects of xenobiotic exposure (8–10). The foci exhibit a proliferative advantage and are thought to represent monoclonal expansions of initiated cells (11). Therefore, investigation of the relationship between proliferation and protection from adduct accumulation in enzyme-altered foci is of interest.

We have investigated cell replication in rat liver during continuous feeding of 0.02% AAF by pulse-labeling with 5-bromodeoxyuridine (BrdUrd) and immunohistochemical staining with anti-BrdUrd. Simultaneous detection of DNA adduct formation has been accomplished with an antiserum specific for *N*-(guanosin-8-yl)-2-aminofluorene (G-C8-AF). Thus, both replication and DNA adduct formation have been studied in the same rat liver cells during continuous carcinogen exposure.

MATERIALS AND METHODS

Animals, Diets, and Tissue Preparation. Young adult male Fisher rats (Charles River Breeding Laboratories), weighing 180–200 g, were kept on a 12-hr light/dark cycle and given water ad libitum. Control rats received a purified, semisynthetic diet (C-1000; Altromin, Lage, F.R.G.). Experimental rats were fed the same diet with AAF (mp 192–196°C; Aldrich) added at a concentration of 0.02% (wt/wt). Rats were fed for 2, 7, 14, 21, or 28 days and received 50 mg of BrdUrd (Sigma) intraperitoneally 1 hr prior to sacrifice by decapitation. Two AAF-fed rats and one control rat were used at each time point. Liver tissue from the median lobe was covered with Tissue-Tec optimal cutting temperature compound (OCT; Miles) and immediately snap frozen in liquid nitrogen. Cryostat sections were cut at 8 μm on a Leitz 1720 cryostat.

Immunohistochemistry. Cryostat sections were processed for paired immunofluorescence staining as described (1). In short, sections were washed for 20 min in cold (4°C) phosphate-buffered saline (PBS), fixed in cold (4°C) methanol/glacial acetic acid, 3:1 for 20 min, and then washed in 70% ethanol twice for 10 min. Next, DNA was denatured by soaking sections sequentially in 70% ethanol with 0.035 M NaOH for 30 sec, twice in 70% ethanol containing 0.1 M Tris (pH 7.6) for 30 sec, and then twice in 70% ethanol for 10 min. Sections were subsequently washed in PBS for 20 min, soaked briefly in deionized water, and air-dried.

Triple fluorescence staining was performed by two-color immunofluorescence for dG-C8-AF adducts and nuclear BrdUrd combined with nuclear Hoechst dye staining. Rabbit anti-G-C8-AF (12) was applied to the sections at a dilution of 1:160 in combination with a mouse monoclonal antibody to BrdUrd (Becton Dickinson) at 1:10 (20-hr incubation at room temperature). After washing, biotinylated horse anti-mouse IgG (Vector Laboratories), containing normal rat serum (1:30) and normal rabbit serum (1:30), was applied at 1:30 for 3 hr. Sections were finally incubated for 30 min with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG (1:20; Dakopatts, Glostrup, Denmark) combined with

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Abbreviations: dG-C8-AF, *N*-(deoxyguanosin-8-yl)-2-aminofluorene; AAF, 2-acetylaminofluorene; BrdUrd, 5-bromodeoxyuridine; G-C8-AF, *N*-(guanosin-8-yl)-2-aminofluorene; FITC, fluorescein isothiocyanate.

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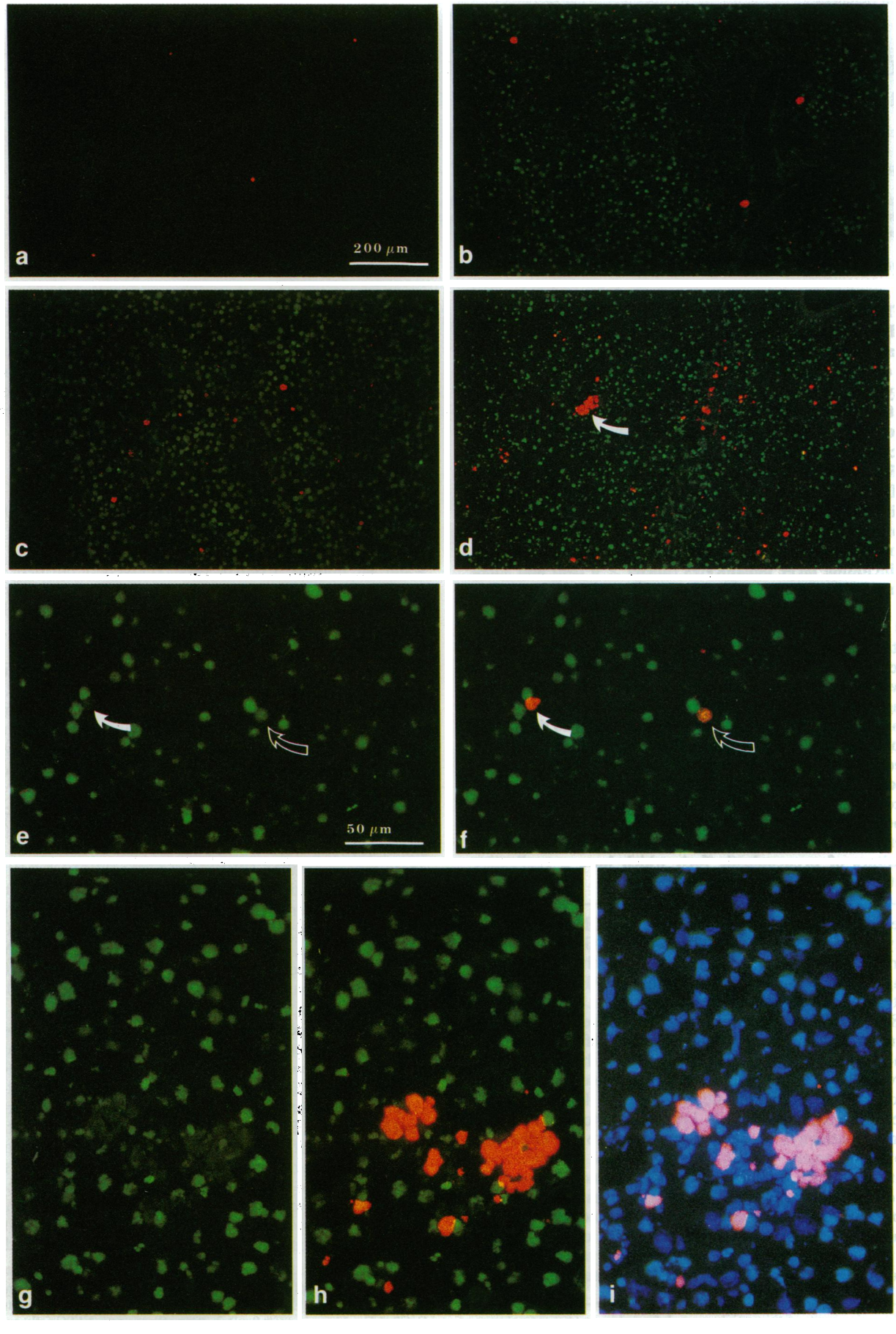

FIG. 1. (*Legend appears at the bottom of the opposite page.*)

Texas Red-conjugated streptavidin (1:200; Bethesda Research Laboratories) containing normal mouse serum (1:30) and normal rat serum (1:30). Sections were subsequently washed for 10 min in PBS containing Hoechst dye (bisbenzimide H 33254 fluorochrome, 0.05 μg/ml; Calbiochem), washed in PBS alone, dipped briefly in deionized water, and mounted with buffered (pH 8.5) polyvinyl alcohol.

Nonspecific staining was evaluated by treating liver sections from both control and AAF-treated rats as described above, except that either rabbit anti-G-C8-AF or mouse monoclonal antibody to BrdUrd was not included in the first antibody mixture.

Microscopy and Photomicrography. Stained sections were examined with a Nikon Labophot microscope equipped with an epifluorescence attachment and filter blocks for FITC and Texas Red as described (1) and a modified UV filter block for Hoechst dye observation. Push-processed (P2) Ektachrome P800/1600 daylight film was used for photomicrography. Combinations of single, double, and triple exposures of the different fluorochromes were used to demonstrate spatial relations between dG-C8-AF adduct content and BrdUrd incorporation in Hoechst dye-stained nuclei.

Scoring of Nuclei Exhibiting dG-C8-AF Adduct or BrdUrd Immunofluorescence. Stained sections were evaluated by the same investigator without prior knowledge of the protocol. An ocular reticle was used for counting cells positive for dG-C8-AF adducts and/or BrdUrd within areas of 0.04 mm^2 of liver tissue. Initially all fluorescent nuclei were included. Thus the observations were obtained for not only large hepatocytes of different ploidy classes but also for hepatocytes with nuclei cut assymetrically (13, 14) and various populations of nonparenchymal cells, including oval cells, Kuppfer cells, and sinusoidal lining cells. Subsequently, in an attempt to observe a less complex cell mixture, the same data were obtained for cells with nuclei >6 μm in diameter. Since the mean nuclear diameter of rat liver parenchymal cells has been shown to be 8.1 μm (13) and even diploid mouse hepatocyte nuclei, which are somewhat smaller, have an average diameter of 6.68 μm (14), it is likely that this size exclusion (6 μm) would result in observations including primarily hepatocytes. In each liver, 20 frames of 0.04 mm^2 were evaluated for each variable, and data from rats of the same group were pooled. As an indication of total DNA content, *all* round Hoechst dye-positive nuclei and those with diameters >6 μm within the 0.04 mm^2 fields were counted. Numbers of adduct-positive nuclei were expressed as a percentage of the total nuclei stained with Hoechst dye.

RESULTS

In frozen sections from livers of male rats continuously fed 0.02% AAF for 1 month, staining for dG-C8-AF adducts demonstrated overall increasing nuclear dG-C8-AF immunofluorescence intensities as well as increasing numbers of positive nuclei during 28 days of AAF feeding (Fig. 1 *a–d*). A preferential periportal distribution of adduct-containing nuclei was observed at all time points, although not all periportal nuclei were stained. Nuclei showing little or no dG-C8-AF immunofluorescence but visualized by Hoechst dye staining were seen predominantly in centrilobular regions in all livers from AAF-fed rats.

In the same frozen sections, cells undergoing DNA synthesis were detected by anti-BrdUrd immunostaining both in control livers and at 2, 7, 14, 21, and 28 days of AAF feeding. During the initial 3 weeks of AAF feeding (Fig. 1 *b* and *c*), such replicating cells were infrequent and randomly distributed, as in control liver (Fig. 1*a*), but at 4 weeks of AAF feeding, the number of BrdUrd-labeled cells had increased substantially (Fig. 1*d*). BrdUrd-labeled nuclei were then found both scattered randomly throughout the liver (Fig. 1*f*) and in discrete clusters (Fig. 1*h*). Almost all of the replicating nuclei of all sizes contained no dG-C8-AF detectable by immunofluorescence, when compared to surrounding non-BrdUrd-labeled liver nuclei (Fig. 1, compare *e* with *f* and *g* with *h*). A few BrdUrd-labeled nuclei contained weak but distinct dG-C8-AF immunofluorescence (Fig. 1 *e–h*). The number of such cells was too low to be determined by the counting method employed. In each of the livers from rats fed AAF for 4 weeks, numerous clusters consisting of up to 20 adjacent BrdUrd-labeled nuclei similarly demonstrated depleted dG-C8-AF adducts (Fig. 1 *d*, *g*, *h*, and *i*).

When nuclei containing dG-C8-AF adducts were counted, increasing numbers of dG-C8-AF-positive nuclei were detected during the initial 14 days of AAF feeding (Fig. 2). The next 14 days of AAF exposure induced no additional increase in the number of dG-C8-AF adduct-containing nuclei. Thus, the kinetics of adduct accumulation appeared similar to those previously reported (2). Simultaneously, the number of BrdUrd-positive cells did not increase substantially during the initial 3 weeks of AAF feeding; however, after 3 weeks the number of cells in DNA synthesis rose strikingly (Fig. 2). By 4 weeks of AAF feeding, the labeling index was 6- to 7-fold higher than in unexposed controls.

DISCUSSION

These results show that during continuous feeding of AAF to male rats, nuclei of liver cells undergoing DNA synthesis contained fewer dG-C8-AF adducts than their surrounding, nonreplicating counterparts. At all times studied during 1 month of AAF feeding, replicating cells generally lacked dG-C8-AF adducts. The number of replicating cells was relatively stable during the first 21 days on the AAF diet but increased notably between 21 and 28 days of feeding. While two main events involved in carcinogenic initiation, carcinogen–DNA adduct formation and DNA replication, have been simultaneously identified at a cellular level in this investigation, the results are limited because the liver is a complex mixture of different cell types. We have been able to observe all nuclei or to focus on liver parenchymal cells by using nuclear size as an inclusion criterion. However, in this study it was not possible to differentiate oval cells, for example, from the cells of many other types having small nuclei.

Previous studies have revealed the lack of dG-C8-AF adduct accumulation in enzyme-altered foci of rat liver induced by four different carcinogenesis protocols when the animals were fed 0.02% AAF for several days prior to sacrifice (3). A recent study, using higher carcinogen concentrations in the diet and

FIG. 1 (*on opposite page*). Frozen liver sections from rats fed the control diet (*a*) or fed 0.02% AAF for 7 (*b*), 14 (*c*), or 28 (*d–i*) days. Sections in *a*, *b*, *c*, *d*, *f*, and *h* were double exposed after staining by paired immunofluorescence for simultaneous identification of dG-C8-AF adducts (FITC) and cells undergoing DNA synthesis (Texas Red) after BrdUrd pulse-labeling 1 hr prior to sacrifice. A single exposure for adducts alone is shown in *e*, and its matching double exposure is shown in *f*. A single exposure for adducts alone is shown in *g* and its matching double and triple exposures are shown in *h* and *i*, respectively. The triple exposure (*i*) included staining for adducts, replicating cells, and total DNA by Hoechst dye (blue emission). Note the occurrence of a replicating focus after 28 days of AAF feeding (*d*, arrow). Photomicrographs in *e* and *f* show a rare replicating cell demonstrating weak dG-C8-AF immunofluorescence (open arrow). Photomicrographs in *g–i* show a focus of cells undergoing DNA synthesis (*h*, Texas Red) with depleted dG-C8-AF immunofluorescence (*g*, FITC) in the focus as compared to surrounding nuclei (*i*, Hoechst dye). (Objective magnification: *a–d*, ×10; *e–i*, ×40.)

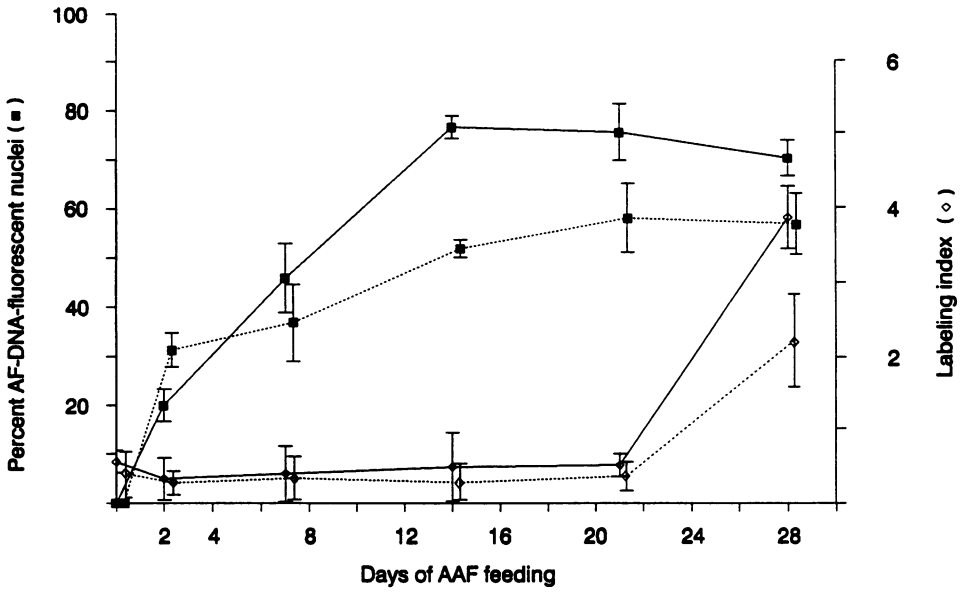

FIG. 2. Nuclei positive for dG-C8-AF adducts and BrdUrd within areas of 0.04 mm^2 of liver tissue from male Fischer rats fed control diet or 0.02% AAF for 2, 7, 14, 21, or 28 days were counted. Left ordinate values are the percent dG-C8-AF-positive cells of all liver nuclei (···■···) or large (diameter >6 μm), putative parenchymal cell nuclei (—■—). Right ordinate values are labeling indices of all (···◇···) and large (diameter >6 μm) (—◇—) nuclei. Values are calculated from the mean ± SD of 20 areas from each rat in each group.

adduct quantitation by the ^{32}P-postlabeling assay, demonstrated adducts in hepatic nodules at much lower concentrations (30–100 fmol/μg of DNA) than those observed in surrounding liver (15). These data do not necessarily conflict with ours, since the lower limit of adduct detection of our immunofluorescence method is 30–50 fmol/μg of DNA (1). The results of both studies are consistent with a general pattern of decreased metabolic and activating (phase I) capabilities and increased detoxifying (phase II) capabilities, which are thought to render these lesions less vulnerable to xenobiotics (4–10). In addition, such putative preneoplastic nodules are considered to have a proliferative advantage. Our present data support the hypothesis that single cells having such biochemical alterations might appear early during continuous carcinogen administration and may represent a subpopulation of hepatocytes that expand more rapidly than normal. Altered metabolism and the capability for increased replication may be two independent events with no causal connection, or one may be made possible by the other.

The growth and differentiation kinetics of the liver are complex and poorly understood. The metabolically less capable neonatal liver grows by replicative mitoses and contains mainly diploid hepatocytes. The normal adult liver is predominantly tetra- and octaploid and grows by polyploidisation (16–18). Since AAF has been shown to inhibit normal hepatocyte regeneration, adduct formation might restrict DNA synthesis, thereby inhibiting growth to a more differentiated, polyploid state. Liver ploidy alterations have been reported during continuous AAF feeding, but no consistent pattern has been demonstrated (19, 20). Generally, preneoplastic and neoplastic liver lesions tend to be diploid, but this is not necessarily universal (21, 22). An understanding of the relationship between growth, ploidy, differentiation, and metabolic capabilities in the liver may elucidate mechanisms of liver carcinogenesis. One may propose that liver cells are protected from adduct formation in an undifferentiated, replicative state and susceptible to adduct formation in a differentiated, nonreplicative state.

In this study, increased hepatocyte replication and the occurrence of proliferative foci were readily detected at 4 weeks of continuous 0.02% AAF feeding. In the same livers, a similar induction of foci positive for placental glutathione-*S*-transferase was observed (unpublished results). Since AAF has been shown to inhibit normal hepatocyte proliferation, it is possible that the increase in cell replication and occurrence of proliferative foci at this time point represents proliferation of initiated cells. The presence of BrdUrd-labeled nuclei as discrete foci is consistent with the concept of clonal expansion of altered cells (11).

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