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Comparison of Proliferating Cell Nuclear Antigen to Tritiated Thymidine As a Marker of Proliferating Hepatocytes in Rats

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Proliferating cell nuclear antigen (PCNA), an endogenous nuclear protein, has recently been used to identify replicating cells. PCNA was compared to tritiated thymidine ([^{3}H]-TdR), a reliable and accurate exogenous labeling agent, to ascertain if PCNA gives comparable results for quantitative cell proliferation. Male F344 rats were treated with a single dose of 500 mg/kg 4-acetylaminofluorene (4-AAF), a known liver mitogen. Rats (n = 5) were euthanized and necropsied at 6, 12, 18, 24, 36, 48, 96, or 192 hr after treatment. Two hours before necropsy, rats were pulse-dosed with [^{3}H]-TdR (2 mCi/kg body weight). Livers were sectioned, autoradiography performed, and labeling indexes (LI), a measurement of the percentage of S-phase hepatocytes, determined. One and a half years after the completion of this study, the archival paraffin blocks of the liver tissue were sectioned and stained for PCNA by an immunohistochemical procedure. Immunocytochemical staining patterns of proliferating cell nuclear antigen expression permitted the recognition of G_1, S, G_2, M, and quiescent cells. PCNA LI, generated by scoring only cells exhibiting S-phase staining patterns, was compared to the pulse [^{3}H]-TdR LI for each animal. Similar periportal staining patterns of S-phase nuclei were detected by both markers. The [^{3}H]-TdR LI and the PCNA LI exhibited a peak at 24 hr of approximately the same magnitude. However, while the [^{3}H]-TdR LI had returned to near baseline at the 48-hr time point, the PCNA LI remained elevated until the 96-hr time point. This sustained elevation of the PCNA index cannot be explained at this time. Examination of all other time points revealed similar S-phase LI by either method. PCNA immunostaining allowed for the estimation of the growth fraction. A time-dependent alteration in the hepatic growth fraction curve was a consequence of 4-AAF treatment.

Introduction

Proliferating cell nuclear antigen (PCNA), an endogenous nuclear protein, has recently been used to identify replicating cells in human and rodent tissues (*1–9*). The PCNA amino acid sequence in the rat and human differs by four residues, indicating a highly conserved molecule (*10*). This 36-kD protein functions as an accessory protein to DNA polymerase (pol) δ (*11,12*). DNA pol δ, in conjunction with PCNA, is also responsible for leading strand DNA synthesis in replicating cells, whereas PCNA involved in DNA repair mechanisms is independent of DNA pol δ (*13*). Studies of cells in culture have identified PCNA in G_1, S, G_2, and M phases of the cell cycle with peak expression of the protein during S phase (*14–16*). The advent of commercially available antibodies of PCNA has allowed the immunohistochemical detection and evaluation of this endogenous cell-cycle-related protein as a marker of replicating cells, which is of particular importance with the recent interest in the role of cell proliferation in chemical carcinogenesis (*17,18*).

Limited data exist that use PCNA for assessment of chemically induced cell proliferation in rodent tissues. The use of PCNA immunohistochemistry in rodent studies depends on its sensitivity, reproducibility, and comparability to other markers of cell proliferation. In this report, we describe *a*) PCNA staining procedures on archival hepatic tissue sections; *b*) the detection of

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cell-cycle-specific phases after PCNA immunostaining; and *c*) a comparison of PCNA to tritiated thymidine ([^{3}H]-TdR) as a marker of chemically induced cell proliferation.

Materials and Methods

Experimental Design

Male F344 rats, 12 weeks of age, were treated with a single dose of 500 mg/kg 4-acetylaminofluorene (4-AAF), a known liver mitogen. Five rats each were euthanized and necropsied at 6, 12, 18, 24, 36, 48, 96, or 192 hr after treatment. Two hours before necropsy, rats were pulse-dosed IP with [^{3}H]-TdR (2 mCi/kg body weight). Livers were fixed in 10% neutral-buffered formalin (NBF) for 2-3 weeks, embedded in paraffin, sectioned, and autoradiography was performed as described by Eldridge et al. (*19*). One and a half years after the completion of the [^{3}H]-TdR study, archival paraffin blocks of the liver tissue were sectioned and stained for PCNA by a immunohistochemical procedure as described by Greenwell et al (*20*). Modifications to this staining procedure, described here in detail, were used to eliminate background staining.

PCNA Staining Procedure

This procedure applied to tissues fixed in 10% NBF for extended periods of time (greater than 72 hr). Liver tissues were sectioned at 6 μm and mounted on tissue adhesive poly-l-lysine coated slides. Sections were air dried, deparaffinized in xylene, and hydrated through a graded series of ethyl alcohols to 1X Automation Buffer, pH 7.5 (Biomeda Corporation, Foster City, CA). A highly sensitive biotin-streptavidin method of immunohistochemical localization was performed as follows (see Table 1 for materials):

1. Quench endogenous peroxidase by placing slides in 3% H_2O_2 for 5 min.
2. Rinse and wash slides in distilled water for 5 min.
3. Place slides in plastic Coplin jar filled with antigen retrieval solution. Microwave on high power (700 watts) for 2 min. Check solution levels after 1 min and repeat high power microwave for 2 min. If levels are decreased, add distilled water.
4. Allow slides to cool for 15 min at room temperature in the Coplin jars.
5. Discard retrieval solution and rinse two times in distilled water.
6. Rinse in 1X automation buffer for 5 min.
7. Apply monoclonal antibody anti-PCNA (19A2). Incubate 30 min at room temperature.
8. Rinse in two changes of 1X automation buffer for 5 min each
9. Apply the link antibody. Incubate for 30 min at room temperature.
10. Rinse in two changes of 1X automation buffer for 5 min each.
11. Apply the label antibody. Incubate for 30 min at room temperature.
12. Rinse in two changes of 1X automation buffer for 5 min each.
13. Rinse slides briefly with distilled water and remove excess liquid from the surface of the slide.
14. Apply diaminobenzidine (DAB) solution to the tissue sections. Incubate 6 min at room temperature in the dark. (Cover chamber and turn out the lights.)
15. Discard excess DAB and rinse slides in running tap water for 3-5 min.
16. Counterstain in Harris hematoxylin (Harelco, Gibbstown, NJ) for 1 min.
17. Rinse in tap water until water is clear.
18. Place slides in 1X automation buffer until sections are blue (3 min).
19. Rinse briefly in tap water.
20. Dehydrate the tissue sections through 95% and 100% ethanol to xylene. Apply Permount (Fisher Scientific Co., Pittsburgh, PA) and coverslip.

Table 1. Materials used for immunohistochemical localization

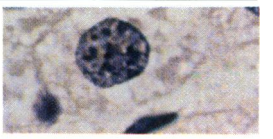
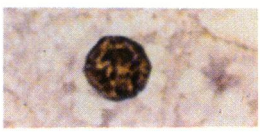
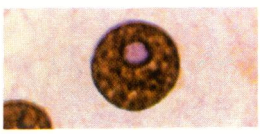
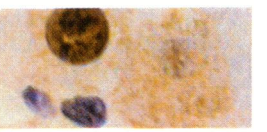
Material (supplier)	Quantity
Reagents	
1X Automation buffer, pH 7.5	
(Biomeda Corporation, Foster City CA)	
10X Automation buffer	100.0 mL
Distilled Water	900.0 mL
Antigen Retrieval Solution	
(Biogenex Laboratories, San Ramon, CA)	
Deionized water	30.0 mL
Antigen Retrieval Solution	10.0 mL
(This is sufficient to fill 1 plastic Coplin jar)	
3% Hydrogen peroxide (make fresh)	
30% Hydrogen peroxide	1.0 mL
Distilled Water	9.0 mL
Substrate-Chromogen Solution	
(may be prepared and stored at 4°C)	
30% Hydrogen peroxide	60.0 μL
Distilled Water	100.0 mL
Add 1 diaminobenzidine (DAB) tablet Sigma Corporation, St. Louis, MO) to 20 mL of substrate reagent 25 min. before using.	
Antibody dilutions	
Primary Antibody Diluent (Prepare fresh)	
Nonfat powdered dry milk	0.5 g
Distilled Water	49.5 mL
Mix well. When ready to use, dilute with equal parts of 1% bovine serum albumin (BSA) in 1X automation buffer.	
Primary antibody: monoclonal antibody mouse anti-PCNA (19A2) (Coulter Immunology, Hialeah FL) (prepare fresh); dilution 1:400	
Monoclonal antibody mouse anti-PCNA	10.0 μL
Primary antibody diluent	3.9 mL
Link and label antibody (StrAviGen TM Super Sensitive Prediluted Immunostaining Kit, Biogenex Laboratories, San Ramon, CA)	
Link	Prediluted
(biotinylated anti-immunoglobulins, specific for mouse)	
Label	Prediluted
(streptavidin conjugated to horseradish peroxidase)	
These kits may be purchased concentrated or prediluted. If using the concentrated kits, the link and label antibody are diluted with 1% BSA in 1X automation buffer at a 1:10 dilution.	

Cell Proliferation Counts

The [^{3}H]-TdR labeling index (LI) for autoradiographed, stained slides was calculated by dividing labeled cells by the total number of cells counted (at least 1000 hepatocytes) from the left lobe of the liver. Nuclei containing at least 10 silver grains were scored as labeled. Binucleated hepatocytes were scored as a single hepatocyte.

For PCNA, the LI and growth fraction were generated for each animal using a serial cut of the same liver section used for determining the [^{3}H]-TdR LI. Immunocytochemical staining patterns of PCNA permitted the recognition of G_1, S, G_2, M, and quiescent (G_0) cells, as described in Table 2. PCNA LI was calculated by scoring hepatocytes exhibiting an S-phase staining pattern. That is, the number of hepatocytes displaying labeled dark brown to black nuclei in the absence of cytoplasmic staining was divided by the total number of hepatocytes counted (at least 1000 hepatocytes). Binucleated hepatocytes were scored as a single hepatocyte. The growth fraction (21), an estimation of hepatocytes active in the cell cycle, was quantitated by dividing the total number of PCNA stained hepatocytes (G_1 + S + G_2 + M) by the total number of hepatocytes counted (at least 1000 hepatocytes).

Table 2. Criteria for phases of cell cycle.

Cell cycle criteria	Positive cell criteria	Example
G_0	No nuclear or cytoplasmic staining.	
G_1	Patchy to uniform, light brown nuclear staining; no cytoplasmic staining.	
S	Uniform, brown-dark brown to black nuclear staining; no cytoplasmic staining.	
G_2	Diffuse, speckled nuclear and cytoplasmic staining.	
M	Diffuse, cytoplasmic staining.	

Results

The modified immunostaining procedure yielded consistent staining patterns, limited background chromogen staining, and allowed the reliable identification of G_1, S, G_2, M, and G_0 hepatocytes. Initial attempts using a previously described procedure (*20*) produced inconsistent staining, increased background, and complications in discriminating cell cycle phases. By reducing the microwave time (i.e., heat exposure), consistent staining was obtained with minimal background. Tissues fixed in formalin for several months required longer microwave incubation to permit optimal expression of the PCNA antigen (data not shown). Since the tissue in this experiment was fixed for a shorter time period (approximately 2 to 3 weeks), decreased microwave irradiation was apparently required to permit select PCNA antigen expression without background staining.

PCNA antigen-antibody complexes expressed various staining patterns. The staining results were categorized based on cellular distribution and intensity of the reaction product as shown in Table 2 and as previously described by Foley et al. (*8*). G_0 or quiescent hepatocytes expressed no detectable staining. G_1 hepatocytes were characterized by a patchy to uniform, light-brown nuclear staining. Cells in S phase displayed uniform intense brown to black nuclear staining. Diffuse speckled-brown nuclear and cytoplasmic staining characterized hepatocytes in the G_2 phase. Mitotic hepatocytes had diffuse and speckled-brown cytoplasmic staining.

The growth fraction revealed that the majority of hepatocytes were G_0 hepatocytes at 6, 12, and 18 hr after 4-AAF treatment (Fig 1). For the next 32 hr, 78% of the cells were active in the cell cycle, dropping to 45% at the 96 hr interval. The proportion of actively cycling cells returned to 3% at 192 hr. The individual cell cycle phases of the growth fraction, as detected by PCNA expression after 4-AAF exposure, are also shown in Figure 1. The proportion of G_1 hepatocytes was elevated after 24 hr (40%) and remained at peak levels through the 96 hr (43%) interval before declining to 3% at 192 hr. S-phase hepatocytes increased after 24 hr (23%) and remained elevated at 36 and 48 hr (17% and 28%, respectively). At 96 and 192 hr, the proportion of S-phase cells had declined to less than 2%. The percentage of actively cycling cells that were G_2- and M-phase hepatocytes was greatest at 36 hr (21% and 4% respectively). At all other time points, less than 1% of the G_2- and M-phase cells accounted for the total percentage of actively cycling hepatocytes. It is evident that 4-AAF treatment greatly altered the hepatocyte growth fraction (Fig. 1 and 2).

Because S-phase hepatocytes were recognized by intense PCNA expression localized in the nucleus, an attempt was made to quantitate these hepatocytes and generate a LI to compare to the previously quantitated [^{3}H]-TdR LI. Figure 3 demonstrates that [^{3}H]-TdR LI and the PCNA LI both exhibited a peak at 24 hr of

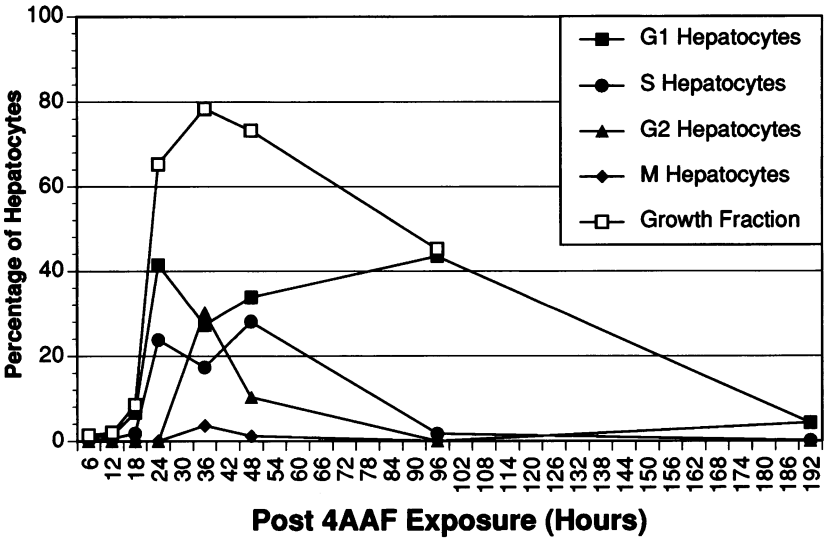


FIGURE 1. The percentage of hepatocytes in various phases of the cell cycle as detected by proliferating cell nuclear antigen after 4-acetylaminofluorene (4-AAF) exposure. The growth fraction displayed a time-dependent alteration as a consequence of 4-AAF treatment.

the same approximate magnitude. However, while the [^{3}H]-TdR LI had returned to near baseline at the 48 hr time point, the PCNA LI remained elevated until the 96-hr time point. Examination of all other time points revealed similar S-phase LI by either methods.

Discussion

An exciting aspect of visualizing replicating cells with PCNA is the potential to assess the degree of chemically-induced cell proliferation in archival material in previously conducted experimental and animal cancer bioassays. Results from these studies indicate a proportional relationship under most conditions between the [^{3}H]-TdR LI and the PCNA LI in the liver of rats treated with 4-AAF. The monoclonal antibody to PCNA (19A2) has also allowed for the estimation of the growth fraction in liver tissue. We observed a time-dependent alteration in the hepatic growth fraction curve as a consequence of 4-AAF treatment. Information gained from the ability to estimate the growth fraction will help with the understanding of cell-cycle kinetics in normal and neoplastic tissue.

Many investigators have attempted to ascertain the validity of PCNA as an indicator of cell proliferation by comparing PCNA to other markers such as [^{3}H]-TdR, BrdU, and Ki67 (*3–5,7,9,22*). Most of the correlations have yielded good results with few exceptions (*7,23,24*). Further investigation is warranted to understand if the discrepancies noted in the exceptions are related to PCNA gene regulation, PCNA expression in different cell types, and/or differences in the antigenicity of anti-PCNA antibodies (*25*). Variables in experimental conditions including the use of different tissue fixatives (formalin versus methanol) may result in a lack of correlation between proliferation markers (*4,7,25–28*). Discrepancies between PCNA and other proliferative cell labeling methods may also result from measuring different indicators of cell division. Proliferative cell labeling markers such as [^{3}H]-TdR and BrdU measure the respective label incorporated into the DNA of cells undergoing DNA synthesis. PCNA as a proliferative cell marker measures the expression of an antigen with a 20 hr half-life that is maximally expressed in S-phase cells.

PCNA, used as an indicator of cell proliferation, has advantages and disadvantages. There is no administration of a labeling compound; therefore, there is no animal surgery, anesthesia, or animal handling. Rapid results are obtained through PCNA immunostaining of retrospective or freshly fixed tissues imparting specific cell cycle stage information. Assimilated information of specific cell-cycle stages allows for growth fraction estimation, yielding more information on a chemical's proliferative effects. PCNA detection can be a disadvantage when quantitating cell replication in a slow turnover tissue. Continuous label administration via osmotic minipumps are required to assess this proliferative data. PCNA LI are more comparable to pulse-dosed [^{3}H]-TdR and BrdU quantitated LI than LI generated by continuous label administration. It is important to remember that PCNA is present in G_1, S, G_2 and M-phase cells. Since all cell-cycle phases are recognizable by PCNA immunostaining, only those cells that meet the positive PCNA scoring criteria as an S-phase cell need to be compared to [^{3}H]-TdR and/or BrdU-S-phase measurements.

In the present study using a monoclonal antibody (19A2) to PCNA, we retrospectively compared the use of PCNA to [^{3}H]-TdR as a marker for detecting S-phase cells. Paraffin blocks of liver from a previously

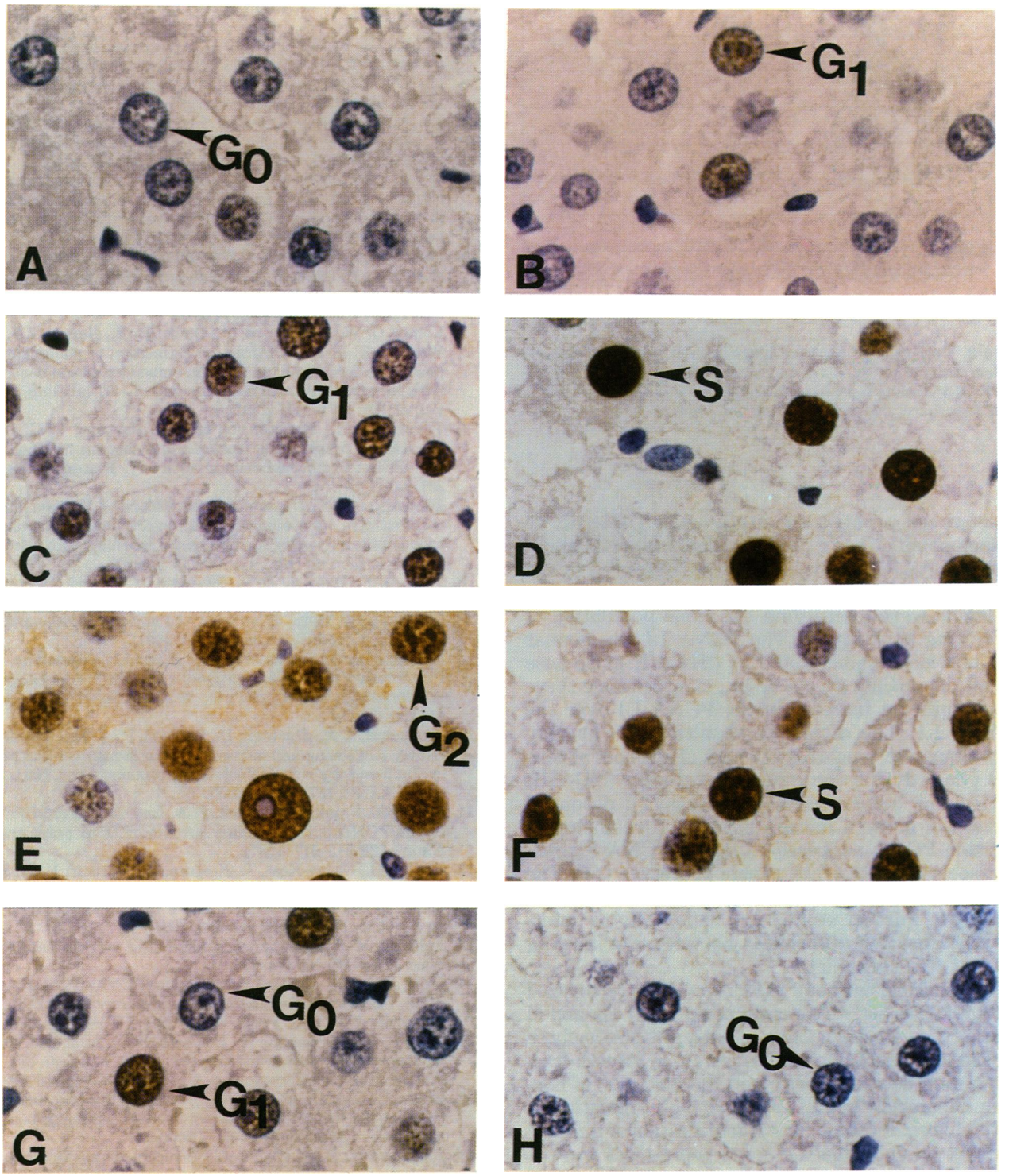


FIGURE 2. Immunohistochemical detection of PCNA using a monoclonal anti-PCNA antibody (19A2). Hepatocytes active in the cell cycle were observed by PCNA immunostaining: (*A*) 6 hr, (*B*) 12 hr, (*C*) 18 hr, (*D*) 24 hr, (*E*) 36 hr, (*F*) 48 hr, (*G*) 96 hr, and (*H*) 192 hr, after 4-acetylaminofluorene treatment. Arrows with the specific cell-cycle phase are adjacent to the hepatocyte displaying the positive cell criteria.

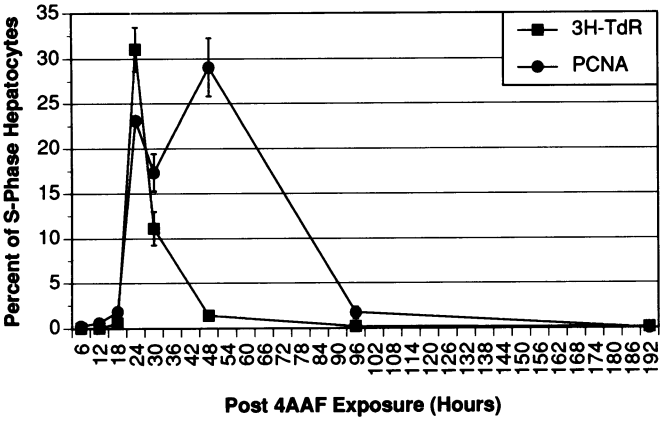


FIGURE 3. Detection of S-phase hepatocytes: proliferating cell nuclear antigen (PCNA) versus tritiated thymidine ([^{3}H] TdR). The PCNA labeling index is comparable to the [^{3}H]-TdR except at 48 hr after 4-acetylaminofluorene administration.

completed cell proliferation study using [^{3}H]-TdR as the labeling agent were used. Similar periportal staining after 4-AAF administration was observed in the PCNA and [^{3}H]-TdR stained slides, suggesting that both labels were identifying the same population of proliferating hepatocytes. The PCNA LI was similar to the previously generated 2 hr [^{3}H]-TdR pulse dose LI at all time points except 48 hr , at which time there was a significant increase in the PCNA LI over the [^{3}H]-TdR LI. The disparate results at 48 hr may reflect a massive synchronous entry of a cohort of cells into S phase during the time interval after [^{3}H]-TdR injection but before tissue sampling (*7*); potential induction of growth factors by 4-AAF resulting in detectable PCNA expression (*25*); or PCNA expression associated with DNA repair processes (*13*). The latter possibility is unlikely since 4-AAF is not genotoxic (*29,30*) Further studies are under way to determine the causative differences between the labeling indexes at this time point.

The monoclonal antibody to PCNA (19A2) recognized an antigen differently expressed in the G_1, S, G_2, and M phases of the cell cycle. Consequently, PCNA analysis was used as an estimator of the cell-cycle growth fraction, yielding new information on the behavior of noncycling hepatocyte populations and their transition to an actively proliferating state. Identification of a chemical's effect on individual cell cycle populations and the total proliferating pool of cells may yield new and potentially valuable information in understanding chemically induced cell proliferation. By adding the specific cell-cycle phases together, it was of interest that approximately 60-80% of hepatocytes were in the active phase of the cell cycle following a single administration of a mitogen. Such analysis may lead to an improved determination of specific cell populations that should be affected by chemical exposure.

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