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Analysis of Proliferating Hepatocytes Using a Monoclonal Antibody Against Proliferating Cell Nuclear Antigen/Cyclin in Embedded Tissues from Various Liver Diseases Fixed in Formaldehyde

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The authors studied histochemically the morphologic features of proliferating hepatocytes positive for proliferating cell nuclear antigen (PCNA/cyclin) to analyze the process of liver regeneration in embedded tissues fixed with formaldehyde using an anti-PCNA/cyclin monoclonal antibody. In liver specimens from patients with acute viral hepatitis (AVH) and confluent necrosis, many small basophilic hepatocytes surrounding large clear hepatocytes were positively stained in the areas next to the confluent necrosis. Therefore these small hepatocytes may be daughter cells derived from large clear hepatocytes that probably enter the mitotic cell cycle repeatedly to repair a large necrotic area. In the case of AVH with spotty necrosis, the positively stained hepatocytes were scattered around the necrotic foci. In the liver specimens from patients with chronic active hepatitis, most of the positively stained hepatocytes were located next to the necrotic area. As for cirrhosis of the liver, the number of hepatocytes positive for PCNA/cyclin varied greatly in different pseudolobules, and in the specimens of hepatocellular carcinoma (HCC), the HCC cells positive for PCNA/cyclin were detected throughout the cancer nests. (Am J Pathol 1992, 140:513–520)

Although it is a well-known fact that the injured liver regenerates rapidly, information on proliferating hepatocytes in the regenerating human liver is scanty. It is not always easy to identify individually damaged hepatocytes from those in the regeneration process, in the injured liver; except when we find hepatocytes with mitotic figures, it is difficult to identify individual hepatocytes as being regenerative.

Recently it has been reported that a monoclonal antibody against proliferating cell nuclear antigen (PCNA/cyclin) could be used to identify proliferating cells by immunochemical methods.[1] PCNA/cyclin is a 36-kd nuclear protein, which increases from late G1 and through the S-phase of the cell cycle. This protein was described independently by Bravo and Celis[2] through two-dimensional gel electrophoretic studies of proliferating and quiescent cells, and by Miyachi et al[3] through the use of human autoantibodies in lupus patients. Furthermore it has been demonstrated that PCNA/cyclin is an auxiliary protein of DNA polymerase delta[4] that plays a critical role in the initiation of cell proliferation.[5] In this study, we confirmed by immunochemical staining using the mouse monoclonal antibody 19F4 against PCNA/cyclin developed by Ogata et al[6] that PCNA/cyclin could be used as a reliable marker of proliferating hepatocytes and hepatocellular carcinoma (HCC) cells. The study was carried out on liver sections fixed in 10% neutral formaldehyde and stocked in paraffin-embedded blocks. The morphologic features of the proliferating hepatocytes and HCC cells identified by this method were examined.

Materials and Methods

Materials

The liver specimens employed in this study were obtained from 22 patients with liver disease diagnosed by

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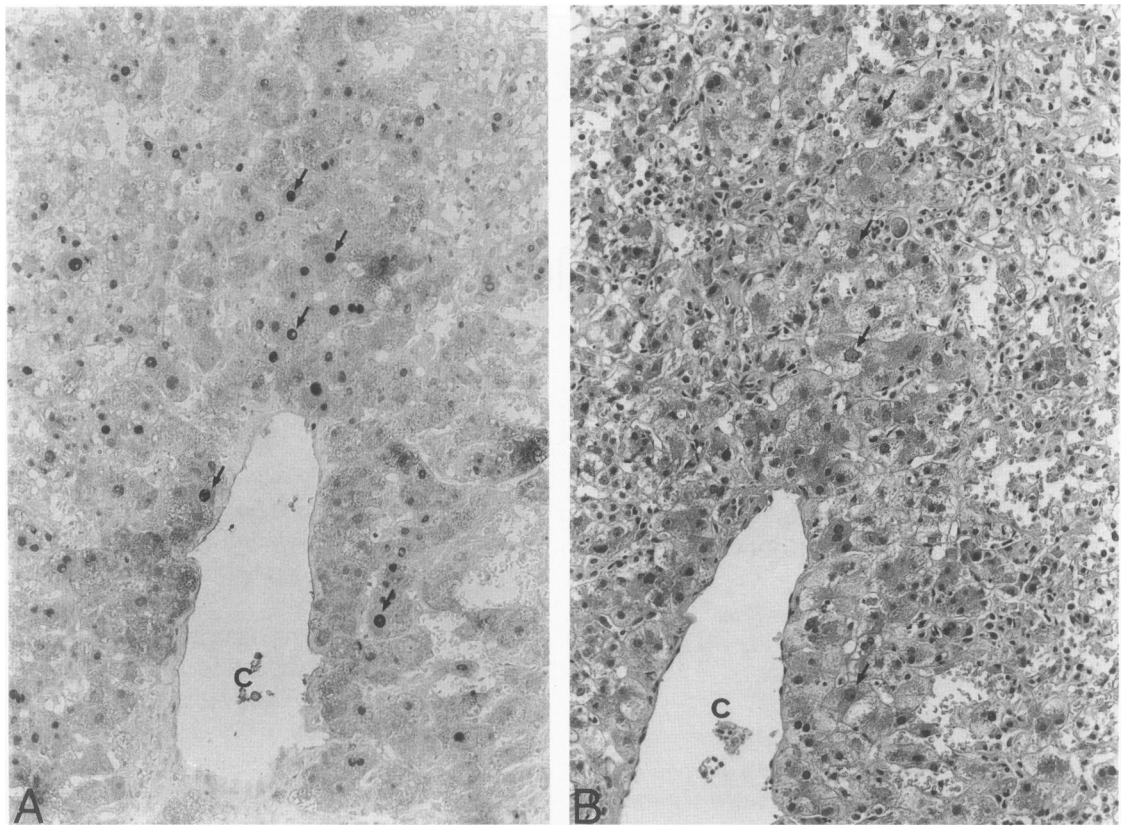

Figure 1. **A:** *Acute viral hepatitis with zonal confluent necrosis. Many hepatocytes positive for PCNA/cyclin (arrows) were found in the area surrounding the zonal confluent necrosis; c, Central vein (fixed in 10% neutral formaldehyde, immunoperoxidase staining method for PCNA/cyclin, counterstaining with methyl green; ×150).* **B:** *Same case as that shown in* **(A)**. *The size and staining of hepatocytes next to the confluent necrosis varied. Many large hepatocytes with clear cytoplasm (arrows) were found, these hepatocytes corresponded to the positively stained hepatocytes in* **(A)**; *C, central vein. (H&E staining, fixed in 10% neutral formalin; ×150).*

clinical tests and by histologic examination. They were eight patients with acute viral hepatitis (AVH) (including one specimen from a second biopsy taken during follow-up of AVH), four patients with chronic hepatitis (CH), five patients with cirrhosis of the liver (CL), three patients with HCC (less than 2 cm in diameter) associated with CL, and two patients with Gilbert's disease (histologically normal controls).

Methods

Fixation and Preparation of the Tissue Section for Immunocytochemistry

The liver specimens were fixed in 10% formaldehyde and embedded in paraffin. Then 6-μ-thick sections were prepared from the paraffin blocks and after deparaffinization through graded ethanol, the sections were washed in phosphate-buffered saline (PBS). At this stage, HCl hydrolysis was done by incubation for 30 minutes at 20°C in 2N HCl, followed by washing in two successive baths (5 minutes each) of 0.1 mol/l borax at pH 8.5. Then the slides were incubated for 10 minutes in 0.5% Tween 20 in PBS and washed twice, 10 minutes each, in PBS.

Immunocytochemical Staining of PCNA/Cyclin

For the immunocytochemical staining of PCNA/cyclin, the avidin-biotin immunoperoxidase method was used. Anti-PCNA/cyclin monoclonal antibody 19F4, a mouse IgG antibody, originally developed by Ogata et al,[6] was obtained from Boehringer Mannheim Yamanouchi Co. (Tokyo, Japan). The tissue sections were first reacted with 10% normal horse serum for 20 minutes at room temperature; then about 100 μl monoclonal antibody 19F4 (concentration: 5 μg/ml) or normal mouse serum (control) was dropped on the tissue sections and allowed to react overnight at 4°C. To inhibit endogenous tissue enzyme activity, the sections were then incubated with methyl alcohol containing 0.3% hydroperoxide, rinsed with PBS five times for 5 minutes each time, and treated with biotinylated horse polyclonal antibody against mouse IgG at room temperature for 2 hours. After rinsing with PBS three times for 5 minutes, the tissue sec-

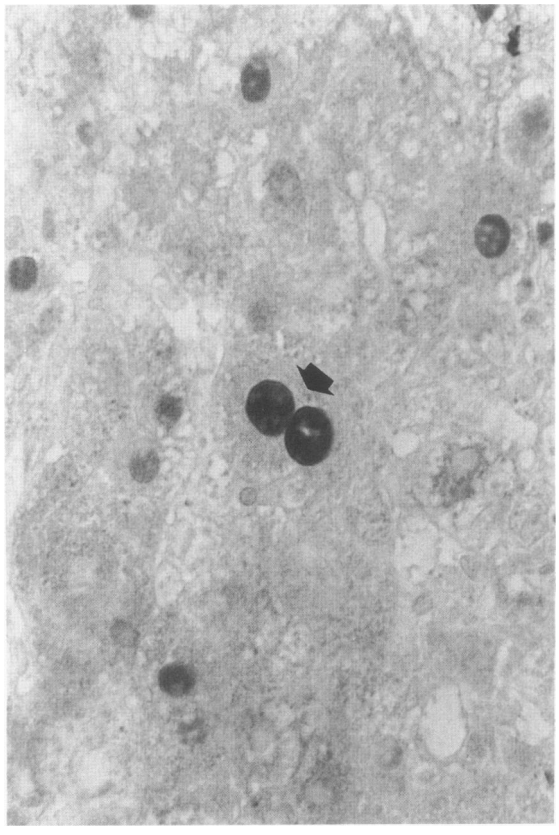

Figure 2. *In the specimens of AVH with zonal necrosis, PCNA/cyclin positive hepatocytes with two nuclei were often detected (arrows) (fixed in 10% neutral formaldehyde, immunoperoxidase staining method for PCNA/cyclin, counterstaining with methyl green; ×600).*

tions were incubated with avidin-biotin-peroxidase complex for 1 hour. Peroxidase activity was demonstrated with 0.025% 3,3′-diaminobenzidine tetrahydrochloride (DAB)-0.003% H_2O_2 in TRIS-buffered saline, pH 7.6, as the substrate. The reaction time was 5 minutes. Then the sections were counterstained with 10% methyl green and mounted with mounting medium. Cells that had reacted with the mouse monoclonal antibody against PCNA/cyclin (positive staining) were stained brown.

Labeling Index for PCNA/Cyclin

The labeling index for PCNA/cyclin, which by definition corresponded to the number of nuclei positive for PCNA/cyclin among 1000 nuclei, was determined for specimens from patients with various liver diseases.

Results

Proliferating cell nuclear antigen/cyclin was detected in the nucleus, which appeared stained in dark brown and exhibited a granular pattern or uniform pattern. In the specimens treated with normal mouse serum and in the specimens obtained from the two patients with Gilbert's disease (nondamaged liver), no hepatocyte nucleus was positive for PCNA/cyclin (all nuclei were stained green). In some biopsy specimens obtained from patients with AVH, zonal confluent necrosis with central to central bridging or central to portal bridging was found. In these specimens, many hepatocytes positive for PCNA/cyclin, which we classified roughly into two groups, were detected. One group consisted of large hepatocytes that had a large irregular nucleus and clear cytoplasm in the sections stained with hematoxylin and eosin (H&E) (Figure 1A, B). In the areas where these large hepatocytes were seen, hepatocytes with two nuclei were often found, and some of these hepatocytes also reacted with the monoclonal antibody against PCNA/cyclin (Figure 2). The other group consisted of small basophilic hepatocytes with a relatively large nucleus and clear nucleolus that were arranged in thick plates (Figure 3A, B). These two types of cells coexisted, especially in the tissue sections with central to portal bridging necrosis. Small basophilic hepatocytes (second group) were found in the areas next to the confluent necrosis, and surrounding large clear hepatocytes and hepatocytes with two nuclei (first group). The number of stained hepatocytes tended to be larger in the second group than in the first group. Furthermore some of the hepatocytes forming rosettes or atypical proliferating ductules in the periportal necrotic areas were also positive for PCNA/cyclin (Figure 4). Conversely in the confluent necrotic area, several interstitial cells also reacted with the monoclonal antibody against PCNA/cyclin (Figure 5). Moreover in the specimens obtained during follow-up for AVH, hydropic hepatocytes arranged with a cobblestone appearance were found, and in these areas many hepatocytes positive for PCNA/cyclin were detected (Figure 6A, B). In the specimens from patients with AVH and spotty necrosis, positively stained hepatocytes were found scattered around the necrosis (Figure 7). In serial sections stained with H&E, these positively stained hepatocytes were of almost normal size and had no characteristic features to be distinguished from unstained hepatocytes. In specimens from patients with CH, hepatocytes positive for PCNA/cyclin were located in the areas next to the active inflammation, for instance, around piecemeal necrosis and bridging necrosis (Figure 8). Most of the positively stained hepatocytes were of normal size, and few small basophilic hepatocytes, which were detected in the specimens of AVH with confluent necrosis, were found. In the specimens from patients with CL, hepatocytes positive for PCNA/cyclin were found mostly in the area next to the fibrotic septum, but the number of PCNA/cyclin-positive cells varied greatly in the different pseudolobules (Figure 9). Positively stained hepatocytes were of various sizes.

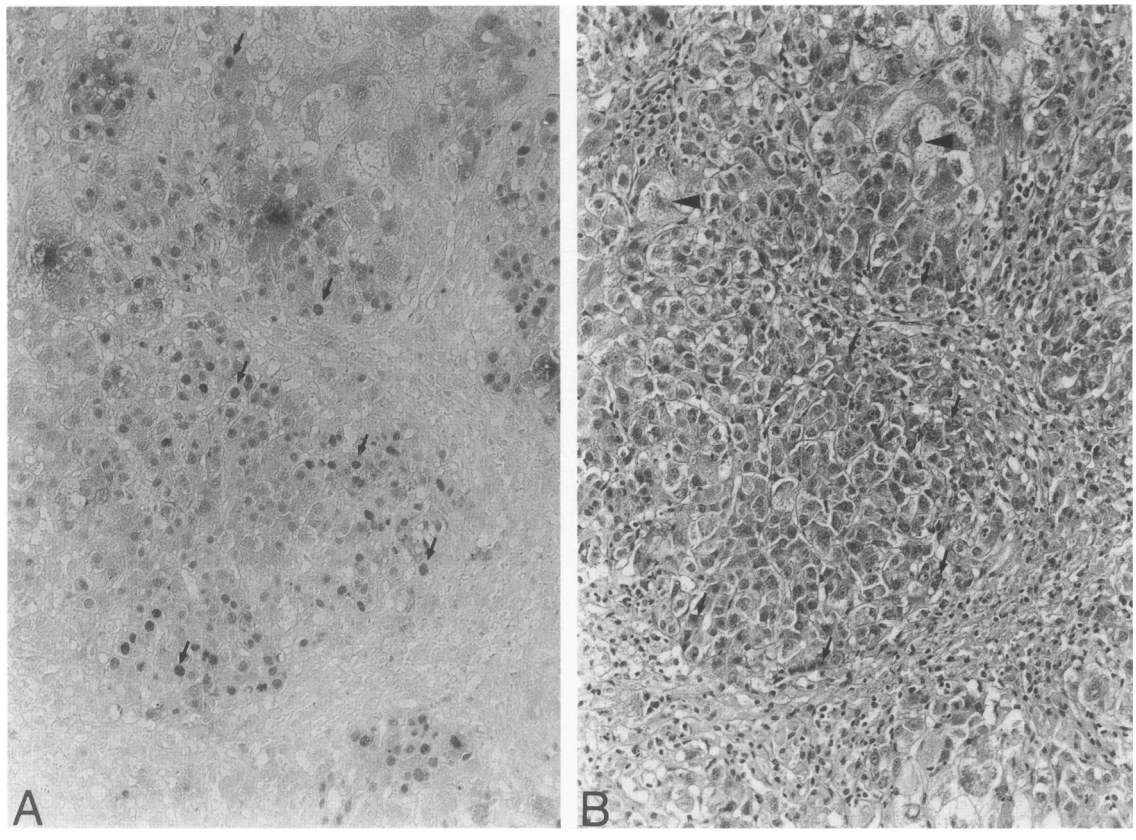

Figure 3. **A:** *Acute hepatitis with zonal confluent necrosis. Many small hepatocytes positive for PCNA/cyclin (arrows) were found in the areas next to the confluent necrosis (fixed in 10% neutral formaldehyde, immunoperoxidase staining method for PCNA/cyclin, counterstaining with methyl green; ×150).* **B:** *A serial section of the same specimen shown in* **(A).** *Many small basophilic hepatocytes (arrows) were found arranged in thick plates, these hepatocytes corresponded to the positively stained hepatocytes in* **(A).** *Moreover, large clear cells (arrowheads) were surrounded by small basophilic hepatocytes (H&E staining, fixed in 10% neutral formalin; ×150).*

In the serial sections stained with H&E, plates two or more cells thick were seen, but the number of positively stained hepatocytes in these areas was not too large. In the tissue specimens from HCC patients, positive HCC cells for PCNA/cyclin were found distributed at random throughout the cancer nests (Figure 10). Generally the HCC cells positive for PCNA/cyclin were small.

The proportion of hepatocytes positive for PCNA/cyclin was estimated under light microscopy by counting of 1000 nuclei in five areas histologically typical of each disease. The mean ± standard deviation was 211.5 ± 215.7 in AVH, 64.0 ± 34.6 in CH, 40.7 ± 19.3 in CL, and 55.6 ± 49.8 in HCC with CL. The proportion of hepatocytes positive for PCNA/cyclin tended to decrease in the following order: AVH > CH > CL (Table 1).

Discussion

Studies on cell kinetics and nucleotide incorporation into the DNA of various tissues are important for the understanding of cell growth, tissue repair after injury, and initiation of malignancy. The incorporation of tritiated thymidine into DNA studied by autoradiography has been used to examine cell kinetics,[7] but this technique is cumbersome and time consuming. A method that uses a monoclonal antibody against bromodeoxyuridine (BrdU) also has been used. However BrdU given as an intravenous infusion occasionally causes reversible toxicity, resulting in myelosuppression or skin photosensitivity, and might become a mutagen.[8,9] Proliferating cell nuclear antigen/cyclin has been proven to be an auxiliary protein of DNA polymerase delta, and it is synthesized in correlation with the proliferative state of the cell.[10–12] Bravo and Macdonald-Bravo[13] have demonstrated that PCNA/cyclin is a stable protein, so it should be detected in quiescent cells at least 24 to 48 hours after cells have stopped dividing. By immunofluorescence studies the existence of two populations of PCNA/cyclin during the S-phase was found; in one, PCNA/cyclin is nucleoplasmic as in quiescent cells and it is easily extracted by detergents, and in the other it is associated with specific nuclear structures. Moreover they also have indicated that by immunoblotting analyses of nonproliferating tissues the amount of PCNA/cyclin detected in those tissues is negligible. Based on these observations, it is thought that PCNA/cyclin exists throughout the cell cycle except for the G0 state and could be a reliable marker of

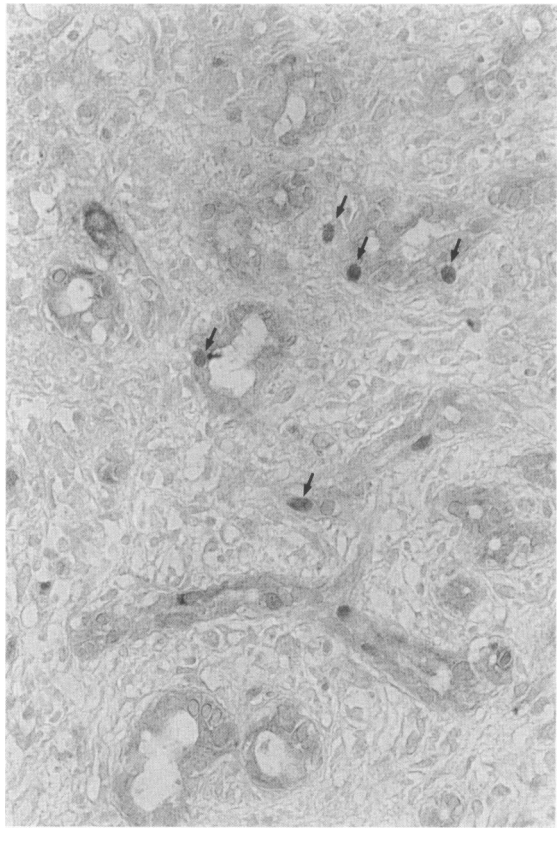

Figure 4. *In the specimens of AVH with confluent necrosis, some of the hepatocytes forming atypical proliferating ductules were positive for PCNA/cyclin (arrows) (fixed in 10% neutral formaldehyde, immunoperoxidase staining method for PCNA/cyclin, counterstaining with methyl green; ×300).*

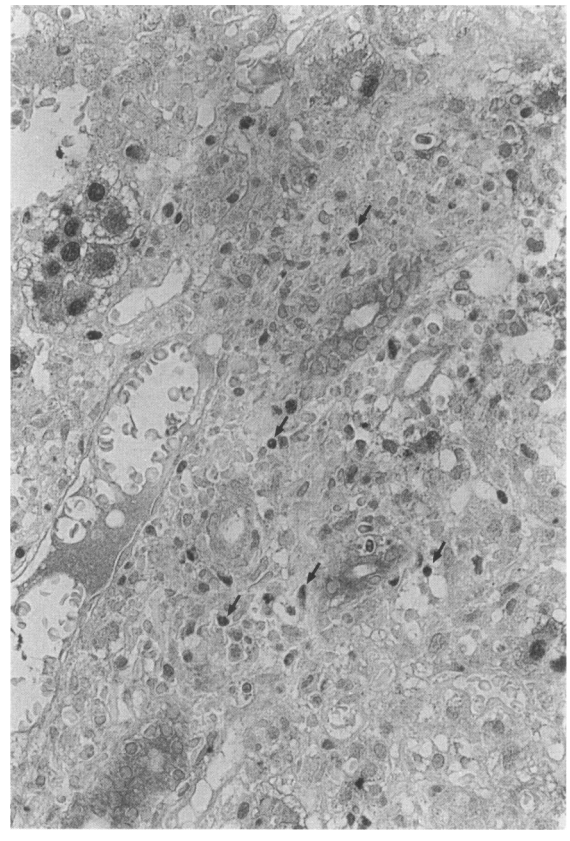

Figure 5. *In zonal necrotic areas of AVH, many interstitial cells were also positively stained (arrows) (fixed in 10% neutral formaldehyde, immunoperoxidase staining method for PCNA/cyclin, counter staining with methyl green; ×300).*

proliferating activity. Although preliminary studies had indicated that anti-PCNA/cyclin antibody could not be used on formaldehyde-fixed tissue, recently Galand and Degraef[14] demonstrated that paraffin sections fixed in formaldehyde and pretreated with 2 N HCl were adaptable for immunocytochemical staining of PCNA/cyclin. We also could detect positive staining for PCNA/cyclin in formaldehyde-fixed specimens from various liver diseases.

Especially in the specimens obtained from patients with AVH and zonal confluent necrosis, a large number of hepatocytes were positively stained for PCNA/cyclin. Many small basophilic hepatocytes that had a relatively large nucleus and were arranged in thick plates were found in the areas next to the confluent necrosis; these hepatocytes were positively stained for PCNA/cyclin. Moreover large hepatocytes with a large irregular nucleus and clear cytoplasm and hepatocytes with two nuclei were found surrounded by small basophilic hepatocytes. Some of these large hepatocytes and hepatocytes with two nuclei were also positively stained for PCNA/cyclin. Based on these findings, we could speculate that small basophilic hepatocytes might be daughter cells derived by cell division of large clear hepatocytes or hepatocytes with two nuclei; these cells would probably undergo several cell cycles, surrounding confluent necrotic areas until repair is achieved. Nostrant et al[15] also have indicated a similar opinion to this speculation in their study of rat liver cell regeneration after selective zonal injury. Moreover in the specimens obtained during follow-up of AVH, hydropic hepatocytes of a cobblestone appearance[16] were seen, and some of these hepatocytes were also positive for PCNA/cyclin. The cobblestone appearance of hydropic hepatocytes could be considered as a phenomenon that occurs because of the filling up of confluent necrotic areas with regenerating hepatocytes. Conversely, positively stained interstitial cells also were detected in the confluent necrotic areas; these interstitial cells may play a role in the repair of liver injury by interacting with parenchymal cells.[17] In the specimens from patients with spotty necrosis, hepatocytes positive for PCNA/cyclin in the vicinity of these necrotic foci were detected. These hepatocytes resembled normal hepatocytes negative for PCNA/cyclin. Similarly in CH, the hepatocytes positive for PCNA/cyclin were mostly in the periportal lesions where hepatic cell necrosis and active inflammation were frequently found. In both AVH and CH specimens, the number of hepatocytes positive for PCNA/cyclin was affected by the extent of the hepatic

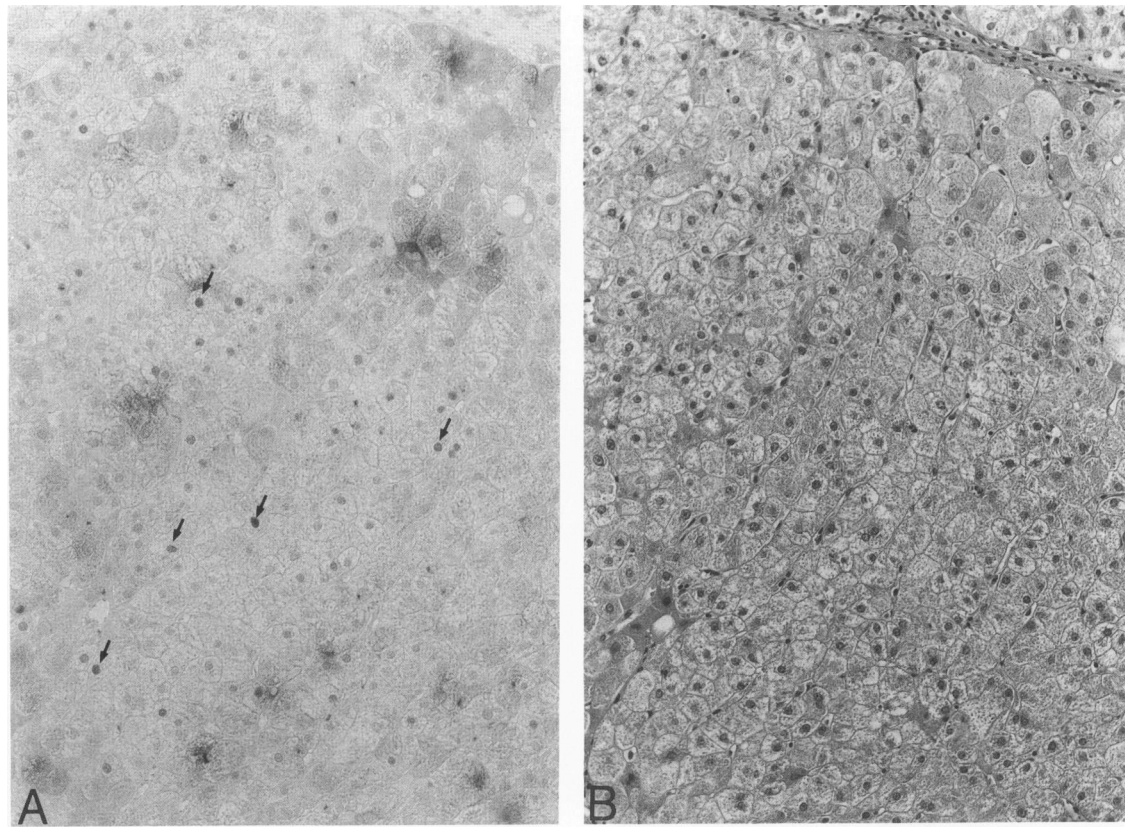

Figure 6. **A**: *In the specimens obtained during follow-up of AVH, hepatocytes positive for PCNA/cyclin (arrows) were detected at random within the lobules (fixed in 10% neutral formaldehyde, immunoperoxidase staining method for PCNA/cyclin, counterstaining with methyl green; ×150).* **B**: *A serial section of the same specimen shown in* (**A**). *Hydropic hepatocytes were found with a cobblestone appearance. These hepatocytes corresponded to the positively stained hepatocytes shown in* (**A**). *(H&E staining, fixed in 10% formalin; ×150).*

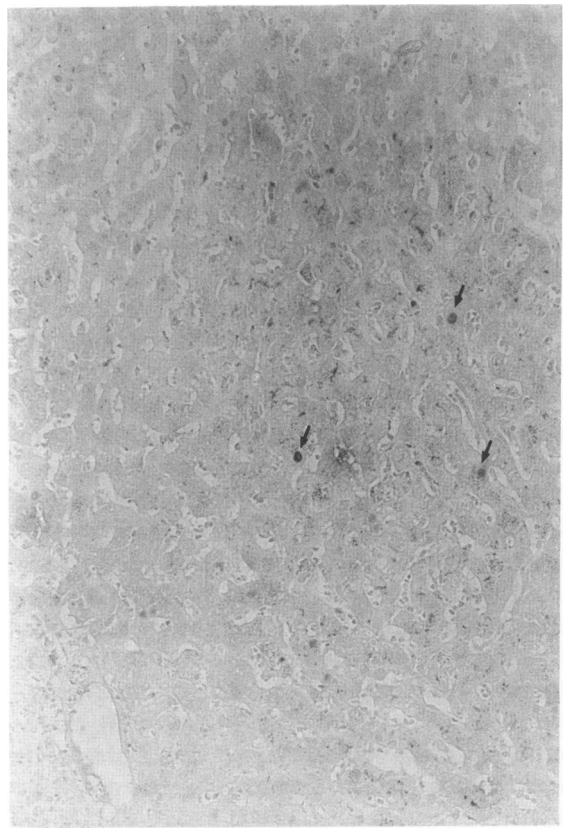

Figure 7. *Acute viral hepatitis with spotty necrosis. Nuclei of hepatocytes positive for PCNA/cyclin (arrows) were found scattered within the lobules (fixed in 10% neutral formaldehyde, immunoperoxidase staining method for PCNA/cyclin, counterstaining with methyl green; ×150).*

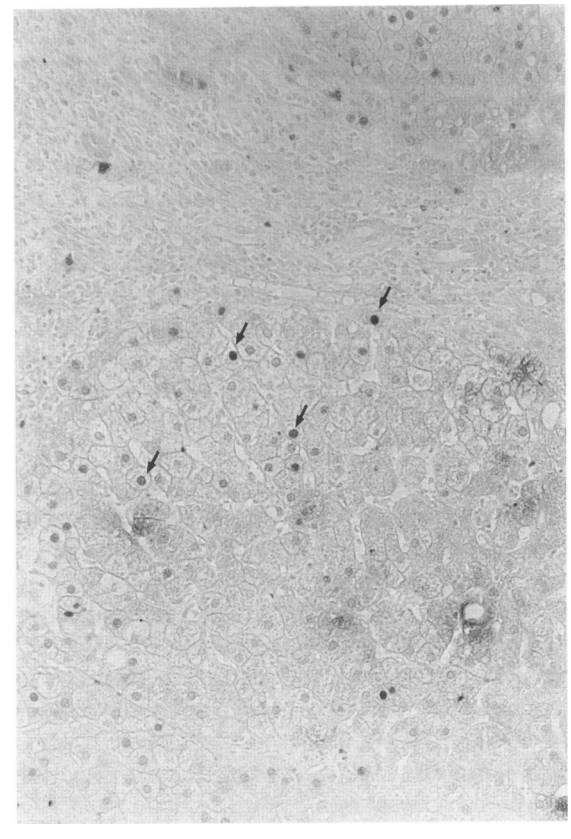

Figure 8. *Chronic aggressive hepatitis. Severe inflammation of mononuclear cells in the portal triad and periportal area was found. Hepatocytes positive for PCNA/cyclin (arrows) were found mainly in the periportal area and next to the bridging necrosis (fixed in 10% neutral formaldehyde, immunoperoxidase staining method, counterstaining with methyl green; ×150).*

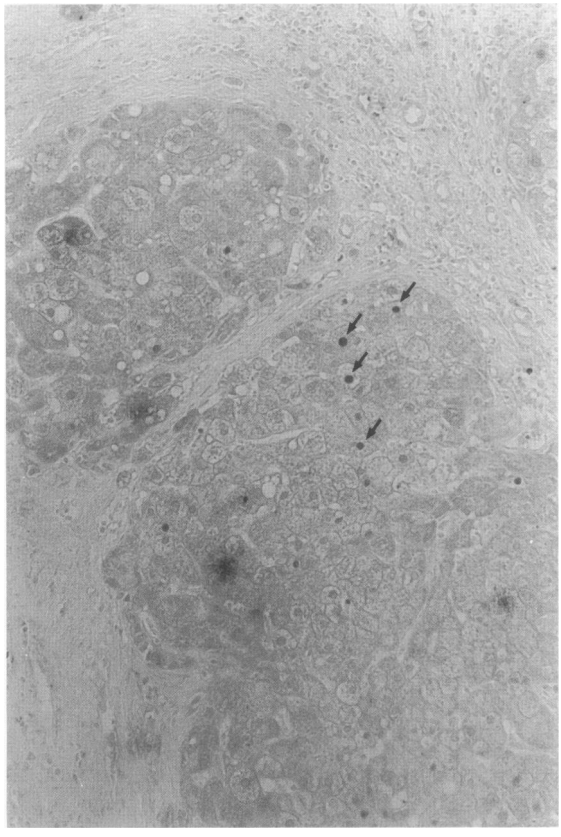

Figure 9. *Cirrhosis of the liver. Hepatocytes positive for PCNA/cyclin (arrows) were found within pseudolobules mostly near the fibrotic septum, but the number of positive hepatocytes varied greatly in the different pseudolobules. (Fixed in 10% neutral formaldehyde, immunoperoxidase staining method, counterstaining with methyl green; ×150).*

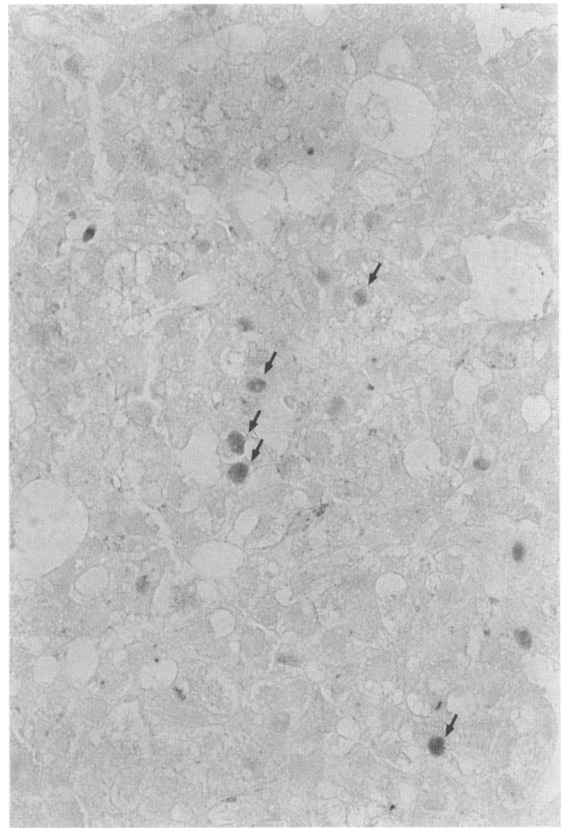

Figure 10. *Hepatocellular carcinoma with cirrhosis of the liver. The hepatocellular carcinoma cells positive for PCNA/cyclin (arrows) were widespread within the center nest. They were found focally, not diffusely (fixed in 10% formaldehyde, immunoperoxidase staining method, counterstaining with methyl green; ×300).*

cell necrosis, but in CH specimens with aggressive inflammation, few small basophilic hepatocytes that reacted with the monoclonal antibody against PCNA/cyclin were also found. Moreover, in CH specimens, the labeling index (LI) of PCNA/cyclin tended to be smaller than in the specimens of AVH. These findings suggest that the progression of liver fibrosis may be related to the lower level of hepatocyte proliferation. In specimens of CL, the number of hepatocytes positive for PCNA/cyclin varied greatly in different pseudolobules, and the negatively stained hepatocytes near the hepatocytes positive for PCNA/cyclin were not always necrotic. These findings may suggest that, in CL, liver regeneration does not begin not only because of local losses of hepatocytes but also because of certain total volume losses of hepatocytes. In specimens of HCC, the positively stained HCC cells were mostly small and sometimes formed foci like islands surrounded by negatively stained HCC cells in the cancer nests. Therefore it is likely that these HCC cells with high proliferating activity replace other HCC cells, and as a result, the grade of malignancy in HCC tissue changes.[18]

Table 1. *Labeling Index of Hepatocytes and Hepatocellular Carcinoma Cells Positive for PCNA/cyclin*

	Labeling index (mean ± SD)
Gilbert's disease (n = 2)	0
Acute viral hepatitis (n = 7)	211.5 ± 215.7
Chronic hepatitis (n = 4)	64.0 ± 34.6
Cirrhosis of the liver (n = 5)	40.7 ± 19.3
Hepatocellular carcinoma (n = 3)	55.6 ± 49.8

Labeling index: The number of nuclei positive for PCNA/cyclin among 1000 nuclei.

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