A survey of liver pathology in needle biopsies from HBsAg and anti-HBe positive individuals

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Abstract

Aims—To use laboratory data and liver biopsies, prospectively obtained from hepatitis B surface antigen (HBsAg) and anti hepatitis B e antigen (anti-HBe) positive patients, for the assessment of: (1) the relation between biopsy length/number of portal tracts and sampling error; (2) the relation between the severity of piecemeal necrosis and the new grading terminology (minimal, mild, moderate, and severe chronic hepatitis); and (3) liver pathology, which has not been studied in patients with this specific serological profile.

Methods—The study group (n = 174) included 104 patients with normal aminotransferase concentrations and no cases with clinically apparent cirrhosis. The specimen length and number of portal tracts were measured at light microscopy examination. Sampling error analysis was related to the discrepancies between aminotransferase concentrations versus histological grade. Detailed histological scorings were undertaken by the reference pathologist and compared with laboratory and hepatitis B virus (HBV) DNA precore sequence data.

Results—Sampling error seemed to be a constant feature, even for biopsies ≥ 20 mm, but increased dramatically in biopsies < 5 mm long and/or containing less than four portal tracts. Between 25% and 30% of biopsies, graded as “mild” or “moderate” activity showed features of moderate and severe piecemeal necrosis, respectively. Ten per cent of the patients with normal aminotransferase values had stage III–IV hepatic fibrosis, and 20% had piecemeal necrosis. Only cytoplasmic, not nuclear, core antigen expression was a strong predictor of high hepatitis B viraemia. There was no association between precore stop codon mutations, grade/stage of liver disease, and hepatitis B core antigen (HBcAg) expression.

Conclusions—The specimen available for light microscopic examination should be > 5 mm long and should contain more than four portal tracts. In addition, the new grading terminology might give the clinician an inappropriately mild impression of the severity of piecemeal necrosis. Furthermore, even in the presence of normal aminotransferase concentrations, considerable liver pathology can be found in 10–20% of HBsAg and anti-HBe positive individuals; such pathology is not associ-

ated with the occurrence of precore stop codon mutations.

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Keywords: liver pathology; chronic hepatitis B virus infection; anti-hepatitis B antigen e positive; core antigen expression; serum hepatitis B virus DNA; hepatitis grading; sampling error

Although the histopathology of chronic hepatitis has, in general, been well described,^{1–3} there has been no specific study of liver pathology in the subgroup of hepatitis B surface antigen (HBsAg) positive patients who are hepatitis B e antigen (HBeAg) negative and anti-HBe positive.

Patients with this serological profile are generally considered to have non-progressive or only slowly progressive chronic liver disease, characterised by low serum hepatitis B virus (HBV) DNA concentrations, low infectivity, and only mild chronic hepatitis.^{4–6} However, previously, such patients may have experienced an HBeAg positive phase of their disease, characterised by active viral replication associated with substantial hepatic necroinflammatory activity.^{7–9} Thus, in the anti-HBe positive subgroup of HBsAg positive patients, a relatively high incidence of advanced hepatic fibrosis associated with a low degree of hepatic inflammation might be expected.

In addition, not every HBsAg positive, anti-HBe positive patient has a remission of hepatitis activity: in 2.8% to 9.2% of HBsAg carriers with anti-HBe antibodies there is appreciable ongoing hepatic inflammation (for example, characterised by piecemeal necrosis).^{7–9} Furthermore, a considerable proportion of HBsAg positive, anti-HBe positive individuals still have actively replicating virus¹⁰ and/or immunohistochemical expression of hepatitis B core antigen (HbcAg).¹¹ There is evidence that progressive chronic liver disease with persistence of high HBV DNA concentrations ($> 10^7$ genomic equivalents (geq)/ml) is associated with HBV strains having mutations in the precore region of the HBV genome. Such mutations create stop codons that abort HBeAg synthesis but leave core protein synthesis, and hence viral replication, unaffected.^{12–14} The most common mutation of this type is a guanosine (G) to adenine (A) substitution at nucleotide (nt) 1896,¹⁵ numbered from the Eco-R1 cleavage site.¹⁶ However, such mutated HBV strains have also been seen in HBV carriers without biochemical evidence of liver disease.^{17–21} Particularly in this subgroup, the relation between the occurrence of such stop

Table 1 Histological scoring of inflammatory activity: Knodell system

Feature	Points
Inflammatory infiltrate in most portal tracts	
None/virtually absent	0
Mild	1
Moderate	3
Severe	4
Destruction of limiting plate by inflammatory infiltrate (piecemeal necrosis)	
None	0
Mild	1
Moderate, < 50% of circumference of most portal tracts	3
Pronounced, > 50% of circumference of most portal tracts	4
Moderate, + bridging necrosis	5
Pronounced, + bridging necrosis	6
Multilobular necrosis	10
Intralobular degeneration (Councilman bodies, ballooning, focal necrosis)	
None	0
Mild (present in < 1/3 of lobules/nodules)	1
Moderate (present in 1/3-2/3 of lobules/nodules)	3
Pronounced (present in > 2/3 of lobules/nodules)	4

codon mutations and histological abnormalities has not been investigated fully.

In HBeAg positive patients, HBcAg expression is associated with active hepatic inflammation,^{6,22} especially when HBcAg is located in the cytoplasm of the hepatocyte.^{23,24} Others have found that only the extent of nuclear HBcAg expression correlated with HBV replication.²⁵ The corresponding relations in the HBeAg negative, anti-HBe positive subgroup appear to have been poorly investigated, although it has been stated that HBcAg is generally undetectable in HBsAg positive, anti-HBe positive patients with HBV DNA concentrations below the detection limit of unamplified hybridisation assays (approximately 10⁷ geq/ml).²⁶ There is also a paucity of data on specific distributions of groundglass cells and immunohistochemical HBsAg expression in relation to hepatitis activity in HBsAg positive, anti-HBe positive patients. In a mixed group of HBeAg positive patients and anti-HBe positive patients, the presence of occasional HBsAg positive hepatocytes scattered throughout the lobule has been associated with active viral replication, whereas large confluent areas of HBsAg positive hepatocytes would be indicative of absent (or very low) viral replication.²⁷ Others did not find an association between specific distributions of HBsAg positive hepatocytes and viral replication or histological activity.²⁸

Apart from the above mentioned, unclarified aspects of chronic liver disease in HBsAg and anti-HBe positive individuals, the availability of laboratory data and liver biopsies, prospectively obtained from HBsAg and anti-HBe positive patients, enabled us to assess two other related issues. First, recommendations for the minimum length of histopathological specimen and number of portal tracts that should be present to provide an acceptably low risk of sampling error vary—for example, 25 mm,²⁹ 20 mm,² 15 mm,³⁰ and 10 mm.³¹ However, Holund *et al* reported that diagnostic sensitivity for piecemeal necrosis was as high as 94% with lengths of biopsy material of only 5 mm.³⁰ The issue is further obscured by the fact that a biopsy of sufficient length (for example, 20 mm) occasionally contains a relatively low number (for example, six) of portal tracts,² which may render it insufficient. Second, since 1994, a

new system has been accepted for grading hepatitis activity, using the terms minimal, mild, moderate, and severe chronic hepatitis.¹ These categories are based on the hepatic inflammatory activity score, which is composed of scores for portal inflammation, piecemeal necrosis, and lobular degeneration as described by Knodell *et al* (table 1). However, epidemiological data have shown that mainly the presence of piecemeal necrosis is associated with progressive chronic liver disease.³² The severity and extent of the piecemeal necrosis might be underestimated using the new grading system. For instance, the presence of “pronounced” piecemeal necrosis (which would have justified the old term “severe chronic active hepatitis”^{33,34}) combined with moderate portal inflammation and a mild amount of lobular degeneration would constitute a hepatic inflammatory activity score of 8 points; according to the new vocabulary, hepatitis activity will be graded as “mild”.

Therefore, we investigated the relation between the extent of piecemeal necrosis and the new hepatitis grading terminology.

Patients and methods

The protocol of our study was approved by the medical ethics committee of each of the participating centres.

From October 1992 to October 1997, HBsAg positive patients in whom HBeAg negativity and anti-HBe positivity had been documented for at least one year were offered a comprehensive assessment of their infection with HBV, including a liver biopsy. Exclusion criteria for our study included lack of written informed consent to undergo a liver biopsy, presence of HBeAg at the time of evaluation or during the previous year, absence of anti-HBe antibodies, consumption of more than 20 alcoholic drinks weekly, antibodies against hepatitis C, δ virus, or human immunodeficiency virus, serious concomitant illness, pregnancy, clinical signs of cirrhosis including ascites, and/or a history of variceal bleeding.

LABORATORY ASSAYS

Serum was assayed for HBsAg, HBeAg, and anti-HBe using an IMx analyser (Abbott Laboratories, North Chicago, Illinois, USA). HBV DNA was assayed using the Chiron branched DNA (bDNA) hybridisation assay (Quantiplex™ HBV DNA assay, Chiron Corporation, Emeryville, California, USA). This bDNA assay has a lower limit of detection of 0.7 Mgeq/ml. If the bDNA assay was negative, HBV DNA was assayed using a validated polymerase chain reaction (PCR) assay,¹⁰ which has a sensitivity of approximately 0.0003 Mgeq/ml. In the statistical analyses, we used the log₁₀ of the serum HBV DNA concentration. HBsAg positivity was confirmed by a neutralisation assay, if serum HBV DNA was not demonstrable by PCR.

SEQUENCE ANALYSIS OF PRECORE AND N-TERMINAL CORE REGION

A nested PCR was used to amplify viral DNA for sequence analysis. DNA was extracted

from serum using the phenol/chloroform method.³⁵ PCR was performed on 10 µl DNA extracts in a 50 µl reaction mixture containing 20 mM Tris HCl (pH 8.4), 50 mM KCl, 1.5 mM Mg²⁺, 0.15 µg of each primer (P₁: 5'-TGGGAGGAGTTGGGGGAGGA, nts 1732–1751, sense; and P₂: 5'-GTC CAAGGAATACTAACATTG, nts 2445–2465, antisense), and 0.2 mM of each of the dNTPs. Forty reaction cycles were carried out as follows: one minute at 94°C, one minute at 55°C, and two minutes at 72°C, followed by 10 minutes at 72°C. Aliquots (2 µl) of the products of the first PCR were subjected to a second round PCR using the same method, but different primers: core 2 (5'-TCTGCG ACGCGGCGATTGAGA, nts 2403–2423, antisense) and P₃ (5'-TGTAACACGACG GCCAGTTAGGAGGCTGTAGGCATAAA TTGGT, nts 1774–1798, sense).

After the second round amplification, DNA samples were purified using the Qiaquick Spin PCR purification kit 2806 (Qiagen GMBH, Hilden, Germany). Aliquots of 60–80 ng of viral DNA were subjected to Taq dye primer cycle sequencing (Applied Biosystems, Perkin Elmer, Foster City, California, USA) using AmpliTaq DNA polymerase FS (Roche Molecular Systems, Alameda, California, USA). Cycle sequencing reaction steps were: 15 × 10 seconds at 95°C, alternating with five seconds at 55°C and one minute at 70°C, followed by 15 × 10 seconds at 96°C, alternating with one minute at 70°C and ending at 4°C. Subsequently, DNA samples were precipitated with 3 M sodium acetate (pH 5.7) in pure ethanol at room temperature in the dark, washed with 80% ethanol, resuspended in 25 µl template suppression reagent (P/W 40167u, Perkin Elmer), denatured for two minutes at 95°C, and loaded on to an ABI prism 310 genetic analyser (Perkin Elmer).

Raw electrophoretic patterns were analysed with the software package Navigator[®] (version 1.0a6, Applied Biosystems, Perkin Elmer) to deduce nucleotide sequences. Nucleotide sequences were subsequently aligned with each other to minimise variation at each genomic position using the Clustal[®] software package (PC-Gene; Oxford Molecular Ltd, Oxford, UK) with a few manual corrections.

HEPATIC HISTOLOGY

A semi-automatic cutting needle (14 gauge/2 mm diameter) was used to obtain most percutaneous liver biopsies. The maximum interval allowed between the laboratory investigations and obtaining a liver biopsy was six months. Specimens were fixed in 4% formaldehyde for at least 12 hours, dehydrated in a series of alcohol solutions of ascending concentration, and embedded in paraffin wax. Sections (4 µm thick) were cut and stained with haematoxylin-azophloxin, periodic acid Schiff after diastase digestion, and Perl's iron stain. Sections were also stained for HBsAg and HBcAg using an anti-HBs monoclonal antibody (Biogenics, San Ramon, California, USA) and an anti-HBcAg polyclonal antibody (Dako, Carpinteria, California, USA), respec-

Table 2 Histological scoring: in house system

Feature	Points
Lobular infiltrate	
None/virtually absent	0
Mild (isolated lymphocytes)	1
Moderate (aggregates and/or rows of lymphocytes)	2
Severe (aggregates of lymphocytes and focal necrosis)	3
Councilman bodies (CBs)	
Number of CBs/10 mm biopsy length ≤ 1	0
Number of CBs/number of portal tracts < 0.3	1
Nber of CBs/number of portal tracts = 0.3–0.7	2
Number of CBs/number of portal tracts > 0.7	3
Fibrosis	
None	0
Mild fibrous extension of portal tracts	1
Porto-portal septa in less than half of the portal tracts	2
Porto-portal septa in more than half of the portal tracts	3
Probable or definite cirrhosis	4
Steatosis	
None	0
Slight	1
Moderate to severe	2
Macrophage reaction	
Virtually absent	0
Mild	1
Moderate	2
Severe	3

tively, followed by peroxidase labelling with streptavidin–biotin–peroxidase complex (Dako).

The study pathologist (FJWTK), who was unaware of the laboratory data, scored portal inflammation, piecemeal necrosis, and lobular degeneration according to the system proposed by Knodell and colleagues² (table 1), as well as several other features, according to an in house developed system, as shown in table 2. A hepatitis activity index was devised based on the sum of points according to table 1, and the severity of chronic hepatitis was graded according to the following hepatitis activity index scores: 1–3, minimal; 4–8, mild; 9–12, moderate; 13–18, severe.¹ The extent of piecemeal necrosis was graded according to table 1 as none, mild, moderate, or pronounced.

We did not apply the grading system proposed by Ishak *et al* because it has not gained wide acceptance in clinical trials since it was published.³⁶

The proportion of hepatocytes that had the appearance of groundglass cells or that stained immunohistochemically for HBsAg, was scored as follows: 0 (0–1%), 1 (1–10%), 2 (10–50%), or 3 (50–100%). The proportion of hepatocyte nuclei that stained immunohistochemically for HBcAg was scored as follows: 0 (absent), 1 (sporadic), 2 (more than sporadic but less than 10%), 3 (10–100%), or 4 (10–100% and cytoplasmic HBcAg expression).

STATISTICAL ANALYSIS

Approximately half of the patients could not recall any specific event that could be related to the beginning of the HBV infection. Thus, the duration of the HBV infection had to be estimated in these cases. For patients originating from countries where vertical transmission is likely, it was assumed that it occurred in the first 5 years of life. For patients originating from Western countries, who were usually

homosexual men, it was assumed that the infection occurred in the first five years after the start of sexual intercourse.

Associations between the various scored histological features and serum HBV DNA concentrations were analysed using the Kruskal-Wallis method.

We developed two statistical measures indicating sampling error, which have not been described previously. These measures are based on the general principle that the finding of a hepatic inflammatory activity score that is unexpectedly low compared with the aminotransferase concentration should raise the suspicion of sampling error.

The first measure was obtained by subtracting the rank number, based on the aminotransferase value (aminotransferase rank), from the rank number, based on the hepatic inflammatory activity score (hepatic inflammatory activity rank): if a patient has a high aminotransferase rank, the hepatic inflammatory activity rank would also be expected to be in the "high area". The occurrence of a large positive difference (aminotransferase rank much higher than hepatic inflammatory activity rank) might indicate sampling error. The opposite, however, is not true, because the finding of an unexpectedly high hepatic inflammatory activity score cannot be the result of sampling error. For this reason, negative differences were set to zero.

The second statistical measure was obtained by calculating a linear regression model that predicted the hepatic inflammatory activity on the basis of aminotransferase values, and looking at the incidence and magnitude of hepatic inflammatory activity scores that were too low compared with the predicted hepatic inflammatory activity (positive residuals). Again, negative residuals were set to zero.

Finally, the occurrence of significant differences between the categories for specimen length and number of portal tracts of the two statistical measures was analysed using the Kruskal-Wallis analysis of variance. The relations between specific mutations and the scored histological features, as well as serum HBV DNA category, were assessed using cross tabulations with Fisher's exact test or the Pearson χ^2 statistic. All statistical analyses were conducted using SPSS software.³⁷

Results

We evaluated 233 HBsAg positive, anti-HBe positive patients. Of these, 193 agreed to further investigations including a liver biopsy. Two anti-HCV positive patients were excluded from the analysis. In six patients, the liver biopsy was not available for examination. In one patient, fibrosis could not be scored because of fragmentation of the biopsy. Biopsies were available for analysis in 184 patients (102 men). Of these, 117 had been detected recently as a result of screening during pregnancy or at blood donation. Most (72%) of the patients were from underdeveloped countries, where HBV infection is endemic and vertical transmission common. The median duration of HBV infection in our study group was

Figure 1 Bar chart showing the effect of biopsy length on sampling error. Two statistical measures, represented by open and hatched bars, were developed that indicate the occurrence of a hepatic activity index score that is unexpectedly low compared with the aminotransferase value (see statistical methods section). The effect of biopsy length on these measures of sampling error, as assessed by the Kruskal-Wallis method, is significant only when biopsies less than 5 mm long are included in the analysis ($p = 0.003-0.03$) but is not significant when biopsies less than 5 mm long are excluded ($p = 0.18-0.51$). Comparable data exist on the number of portal tracts in each biopsy.

estimated to be 27 years, and 90% of patients had an estimated duration of HBV infection that exceeded 13 years.

The median interval between the laboratory tests and the liver biopsy was 13 days, and in 90% of patients these intervals were less than 55 days.

Figure 1 shows the influence of biopsy length on our measures of sampling error. Based on these data, 10 biopsies less than 5 mm long and/or containing less than four portal tracts were excluded from further analysis. Thus, a total of 174 liver biopsies was studied.

Cirrhosis was found on histological examination in nine patients (5.2%). Severe hepatic fibrosis (stage III) was found in another 22 patients (12.6%). In patients with normal aminotransferase values ($n = 104$), piecemeal necrosis was present in 20 (20%) and stage III-IV hepatic fibrosis in 10 (10%).

Table 3 provides a comparison of the severity of piecemeal necrosis and the hepatitis grade.

On further analysis of the scored histological features, close associations were found between portal inflammation, piecemeal necrosis, and fibrosis (p values < 0.00005), and between Councilman bodies and lobular inflammation (table 4). Indeed, in 25% of the patients lobular inflammatory activity was not associated with portal or periportal inflammation. The most significant inter-relations between scored histological features are illustrated in fig 2, in which the thickness of the connecting lines corresponds to the strength of the associations.

Table 3 A comparison between the severity of piecemeal necrosis and hepatitis grade

Piecemeal necrosis	Chronic hepatitis activity			
	None	Minimal	Mild	Moderate
None	40	59	11	
Mild		27	18	
Moderate			8	6
Pronounced (severe)			3	2

Table 4 Kruskal-Wallis analysis of variance of inter-related histological scores

	Portal inflammation by..	Piecemeal necrosis by..	Lobular inflammation by..	Councilman bodies by..	Macrophage reaction by	Fibrosis stage by..	HBcAg by..	Ground glass cells by..	Steatosis by..	Log serum HBV DNA by..
Portal inflammation		85.1	35.2	41.5	10.8	54.1	35.2	5.9	5.5	24.5
Piecemeal necrosis	80.6	<0.00005	<0.00005	<0.00005	0.0127	<0.00005	<0.00005	0.1150	0.1404	<0.00005
Lobular inflammation	32.8	15.3	14.0	29.9	5.9	78.5	42.5	11.4	2.2	26.6
Councilman bodies	43.1	30.0	80.2	80.3	8.9	5.6	<0.00005	0.0097	0.5283	<0.00005
Macrophage reaction	8.4	6.2	7.9	8.9	0.0305	0.1339	0.0547	0.0414	0.1079	0.0259
Fibrosis stage	56.2	80.4	17.3	20.9	6.1	15.9	25.6	8.9	4.8	12.8
HBcAg expression	34.4	41.8	7.8	22.7	7.7	21.8	0.0003	0.0311	0.1850	0.0050
Ground glass cells	5.8	8.8	4.5	4.7	1.4	5.9	5.2	1.1	2.0	0.0763
Steatosis	6.8	9.2	4.5	6.9	1.1	6.1	3.7	1.3	6.11	0.1561
	0.0330	0.0099	0.1064	0.0314	0.5686	0.0467	0.1568	0.5210	0.0471	

The numbers denote the significance levels of the χ^2 statistic, corrected for ties. The row variables are the grouping variables, the columns the dependent variables. For ground glass cells the patients with a score of 1 are compared with the other patients (scores 0, 2, or 3). HBcAg, hepatitis B core antigen; HBV, hepatitis B virus.

Advanced (stage III) hepatic fibrosis in the absence of piecemeal necrosis was encountered infrequently in our study group—84% of the patients with stage III–IV hepatic fibrosis showed features of piecemeal necrosis as well. One hundred and ten patients showed no features of piecemeal necrosis. Of these, only five patients (4.5%) had stage III–IV hepatic fibrosis.

HBcAg EXPRESSION, HBV DNA VALUES, AND HEPATITIS ACTIVITY

Sections stained immunohistochemically for HBcAg were not available in nine cases. Scores for HBcAg expression were strongly associated with those for portal inflammation, piecemeal

necrosis, Councilman bodies, fibrosis, and serum HBV DNA values, and to a lesser extent with those for lobular inflammation (table 4).

Strong HBcAg expression (score 3 or 4) was found in 11 of 165 patients (6.7%); eight of this subgroup (4%) had cytoplasmic expression of HBcAg. Mild HBcAg expression (score 2) was found in nine patients. In 20 cases there was only sporadic expression of HBcAg in hepatocyte nuclei. Thus, despite serum being negative for HBeAg and positive for anti-HBe in all cases, HBcAg expression was found in 40 of 165 patients (24%).

A comparison between serum HBV DNA values and HBcAg expression could be made in 145 patients, and in 35 of these patients HBcAg expression was detected immunohistochemically. Figure 3 illustrates the relation between serum HBV DNA values and HBcAg expression. No HBcAg expression was de-

Figure 2 Graphic representation of the strongest associations ($p < 0.0005$) that were found between the most important scored histological features. The thickness of the connecting lines corresponds to the χ^2 values in table 4. The strongest associations are in the triangle portal inflammation–piecemeal necrosis–fibrosis, and between lobular inflammation and Councilman bodies. HBcAg, hepatitis B core antigen.

Figure 3 Bar chart representing the proportions of patients that were found to have certain levels of HBcAg expression, according to groups defined by serum HBV DNA values. HBcAg, hepatitis B core antigen; HBV, hepatitis B virus.

Figure 4 Bar chart representing cross tabulations of scored histological features (portal inflammation, piecemeal necrosis, hepatic fibrosis, and core antigen expression), and serum hepatitis B virus (HBV) DNA category for patients without (upper bar) or with (lower bar) a G → A mutation at nucleotide 1896. The G → A mutation creates the well known stop codon that is thought to be associated with more aggressive chronic liver disease. bDNA, branched DNA.

tected in 19 of 36 patients with a positive bDNA assay. Interestingly, in one patient with sporadic nuclear staining for HBcAg, HBV DNA could not be detected in serum by PCR. Furthermore, in 17 of the 27 patients in whom nuclear, but not cytoplasmic, HBcAg was detected, serum HBV DNA was only demonstrable by the PCR assay. However, all eight patients with cytoplasmic HBcAg expression had serum HBV DNA values that were detectable by the bDNA assay.

There was one patient with serum HBV DNA values above 10 Mgeq/ml in whom no HBcAg was detectable in the biopsy. Because this patient had cirrhosis, this was probably the result of sampling error.

PRECORE MUTATIONS AND HISTOLOGICAL FEATURES

Owing to restricted logistic capacity, sequence analysis of the precore region was done for the first 80 patients who were enrolled in the study. At the time of the final analysis, there were no significant differences found in mean or median aminotransferase concentrations ($p = 0.99$), mean or median hepatic activity

index ($p = 0.81$), or median serum HBV DNA values ($p = 0.19$) between patients in whom nucleotide sequencing was performed and those in whom sequencing was not done. In 54 patients, precore translation was found to be aborted by a mutation in the precore region. In 40 patients, this was the result of the classic stop codon created by the G to A mutation at position 1896 of the HBV genome.¹⁵ There were no significant differences in aminotransferase values ($p > 0.85$), serum HBV DNA values, or histologically scored features between patients with or without the nucleotide 1896 G to A mutation (fig 4).

GROUNDGLASS CELL PATTERNS AND HBsAg EXPRESSION

Sections stained immunohistochemically for HBsAg were not available in 13 cases. Patients with the highest (3) or the lowest (0) scores for groundglass cells usually had very low inflammatory activity and no evidence of active viral replication. Typically, groundglass cells appeared as large confluent areas, separated by areas of hepatocytes that did not express HBsAg.

Patients with a score of 1 for groundglass cells had significantly higher histological scores for inflammatory features than patients with a score of 0, 2, or 3 (table 4). In these patients, groundglass cells were scattered throughout the lobular parenchyma, which often had a disturbed architecture.

The scores for immunohistochemical HBsAg expression were, on average, higher than the corresponding scores for groundglass cells. This finding was attributable to hepatocytes with low level HBsAg expression not having a groundglass cell appearance. For instance, of the 53 patients with a score of 0 for groundglass cells, 39 had immunohistochemical evidence of HBsAg expression. We could not, however, find a significant association between scores for HBsAg expression and histological inflammatory features, although patients with a score of 1 for HBsAg expression had higher scores for fibrosis than patients with a score of 0, 2, or 3 for HBsAg expression ($\chi^2 = 9.4$; $p = 0.03$). In addition, the differences between the scores for HBsAg expression and groundglass cell density, reflecting the extent of low level HBsAg expression, did not correlate with the histological scores of either inflammatory activity or fibrosis (data not shown).

MISCELLANEOUS HISTOLOGICAL FINDINGS

Mild steatosis was found in 73 patients (42%) and moderate steatosis in 20 patients (12%). There were weak associations between the degree of steatosis and the histological scores for inflammation and fibrosis, with the exception of the score for lobular inflammation. Steatogranulomas were found in 11 patients and did not correlate with any of the histological scores for inflammation. Mild to moderate features of cholangitis, such as distorted bile duct epithelium accompanied by intraepithelial lymphocytes,^{38,39} were found in 21 patients (12%); in eight of these patients there was mild

and occasionally moderate pericholangiolar fibrosis as well. These bile duct abnormalities did not correlate with any of the histological scores for inflammation or fibrosis, but the number of patients with bile duct abnormalities might have been too low to demonstrate such associations.

Slight intrahepatocytic iron accumulation was found in three patients. Two patients had focal subendothelial iron depositions, which might have been a consequence of long standing periportal hepatocyte necrosis.⁴⁰ Portal lymph follicles were found in another three patients.⁴¹

Discussion

Our study investigated three issues: sampling error, grading, and histopathology in HBsAg and anti-HBe positive patients. We will discuss these issues separately.

With regard to the first issue, our newly developed statistical measures indicate that the risk of sampling error is a relatively constant feature for specimens from 5 to over 20 mm length and/or containing more than four portal tracts. Indeed, this risk does not seem to decrease in long biopsies. This might be attributed to the fact that even a large biopsy is only 1/100 000th of the total liver.⁴² However, if the specimen is shorter than 5 mm and/or contains less than four portal tracts, the risk of sampling error increases dramatically.

When assessing the relation between the new terminology for grading hepatic necroinflammatory activity and the extent of piecemeal necrosis (table 3), it was found that the grading system might give the clinician an impression of hepatitis activity that is inappropriately mild. Indeed, 7.5% of cases classified as "mild" chronic hepatitis in reality showed features of severe piecemeal necrosis, and an additional 20% had moderate piecemeal necrosis. Although the number of patients with "moderate activity" was low, a quarter of these patients in reality had severe piecemeal necrosis.

Because piecemeal necrosis is one of the strongest risk factors for progressive chronic liver disease,³² we would suggest that, in addition to the new grading terminology, presence, extent, and activity of piecemeal necrosis is mentioned separately in the pathologist's conclusion, to prevent the impression of hepatitis activity being inappropriately mild.

The specific histopathology that can be found in HBsAg and anti-HBe positive patients has not been described previously, perhaps because most physicians believe that such patients do not have chronic progressive liver disease when aminotransferase values are within the normal range.

Our study demonstrates, however, that a wide spectrum of chronic hepatitis activity occurs and that aminotransferase values may tend to underestimate the severity of chronic hepatitis and hepatic fibrosis in patients with this serological profile. Indeed, 20% of the patients with normal aminotransferase values had piecemeal necrosis and 10% had severe hepatic fibrosis or cirrhosis. We developed a logistic regression model that enables the clinician

to estimate the chance of finding chronic active hepatitis on liver biopsy, based on the plasma aminotransferase value only. This model demonstrates that if the plasma aminotransferase value is greater than 0.5 times the upper limit of normal, the chance of finding chronic active hepatitis on histological examination is greater than 50%.⁴³

Of note, is the fact that we did not find an association between the presence of precore mutations, severity of liver disease, and viral replication parameters (serum HBV DNA and HBcAg expression). These findings are consistent with the results of five earlier reports,¹⁷⁻²¹ which suggested that precore mutations in HBV that block HBeAg synthesis, especially the nt G1896A stop codon mutation, do not by themselves enhance chronic hepatitis activity or HBV replication.

Thus, a more cautious approach to HBsAg positive, anti-HBe positive patients is justified, and a liver biopsy should probably be taken more often to elucidate the true hepatitis activity and fibrosis stage. The presence of chronic active liver disease in an HBsAg and anti-HBe positive patient does not necessarily imply infection by a precore mutant HBV strain.

Because antiviral treatment is still of limited success, the main reason for histological examination in an HBsAg and anti-HBe positive patient is the detection of clinically asymptomatic advanced hepatic fibrosis (stage III-IV), which puts the patient at particular risk for the development of hepatocellular carcinoma.⁴⁴ Indeed, in these patients, three monthly determinations of serum α -fetoprotein concentrations and liver ultrasound examinations have been recommended,^{45 46} and probably will improve survival.⁴⁷

According to our data, current "routine" immunohistological staining procedures for hepatitis B antigens are not particularly useful. Given the fact that a diagnosis of hepatitis B virus infection has already been made on the basis of positive hepatitis B serology, confirmatory immunohistochemical staining for HBsAg is obviously unnecessary. Although we found a weak association between a "scattered" appearance of HbsAg positive cells and more active disease, this morphological pattern is of limited practical value.

Regarding the immunohistochemical detection of HBcAg, it seems that measurements of serum HBV DNA concentrations are superior as an indicator of HBV replication, at least when compared with routine immunohistochemical HBcAg staining.

In our data, the absence of immunohistochemically stainable HBcAg did not exclude high serum HBV DNA values (> 0.7 Mgeq/ml), and neither did the detection of HBcAg in the hepatocyte nucleus reliably indicate active viral replication (> 0.7 Mgeq/ml). Indeed, in 10% of patients in whom serum HBV DNA was undetectable by PCR, a few hepatocyte nuclei stained positive for HBcAg! In contrast, the presence of cytoplasmic expression of HBcAg was always accompanied by serum HBV DNA values greater than 0.7 Mgeq/ml.

A last word should be said about the hepatitis activity index, which sums the histological scores for portal inflammation, piecemeal necrosis, and lobular degeneration. What exactly is the basis of these individually scored histological features and what is the prognostic relevance of the individual components and the total score? All of these questions are not yet answered. We found a cluster of strong associations between portal inflammation, piecemeal necrosis, and fibrosis, and a second cluster of associations between lobular inflammation and Councilman bodies. It might be that the "portal" cluster is related to CD4 lymphocytic activity, whereas the "lobular" cluster is related to CD8 lymphocytic activity.⁴¹ If this is true, it would be worthwhile to investigate further the separate relations between portal (portal inflammation plus piecemeal necrosis plus fibrosis) and lobular (lobular inflammation plus Councilman bodies) indices and the long term progression of liver disease in HBsAg positive, anti-HBe positive patients.

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