Virchows Archiv (1994) 424:465–469


ORIGINAL ARTICLE

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# Immunoperoxidase staining of hepatitis C virus in formalin-fixed, paraffin-embedded needle liver biopsies

Received: 18 November 1993 / Accepted: 22 February 1994

**Abstract** The localization of hepatitis C virus (HCV) in the liver has not been well clarified. We report successful indirect immunoperoxidase staining of the HCV core antigen using polyclonal antibodies raised in rabbits and conventional formalin-fixed, paraffin-embedded needle biopsy sections of liver. The core antigen was distributed in a fine granular pattern diffusely, perisinusoidally, or focally within the hepatocellular cytoplasm of livers from patients with HCV infection. The staining tended to show a more heterogeneous pattern in terms of intensity and distribution in cases of more advanced disease. Hepatocellular carcinoma cells were also frequently stained. HCV immunostaining will provide important information on the pathogenesis and treatment of HCV-related liver diseases.

**Key words** Viral hepatitis · Hepatitis virus · Hepatitis C
Hepatocellular carcinoma · Immunostaining

## Introduction

Since the successful cloning of the hepatitis C virus (HCV) genome [2] and subsequent development of diagnostic kits for HCV [10], great advances in HCV research have been achieved. The structure of the HCV gene [8] and its genotypic diversity [3] have been clarified and putative virus particles visualized [1].

Attempts have also been made to specify HCV-related antigens by immunostaining [5, 9] and HCV RNA by in situ hybridization [11, 14] in liver tissue. However, cryostat sections used for the former technique are troublesome to prepare and inadequate for precise observation, and the latter technique is not easy.

In order to overcome these drawbacks, we carried out immunostaining of HCV using formalin-fixed, paraffin-embedded sections of liver tissue, since clarification of HCV localization in the liver would provide deeper insights into the pathogenesis of liver diseases. It was also anticipated that immunostaining would yield information on the kinds of cells harbouring the replicating virus. Attempts to stain HCV immunologically in paraffin sections have been few [7].

## Materials and methods

A total of 104 patients were selected randomly from the file used for registration of liver biopsies at four institutes in 1992 and 1993. The patients were classified clinicopathologically into the following categories: (i) 10 patients (25–48-years-old, 5 males) with acute hepatitis C, (ii) 40 patients (27–63-years-old, 29 males) with chronic hepatitis C, (iii) 8 patients (45–72-years-old, 6 males) with HCV-related liver cirrhosis, (iv) 6 patients (57–73-years-old, 6 males) with HCV-related hepatocellular carcinoma (HCC) arising in cirrhosis, (v) 5 patients (22–39-years-old, 3 males) with acute hepatitis B, (vi) 20 patients (26–53-years-old, 14 males) with chronic hepatitis B, and (vi) 15 patients (32–55-years-old, 15 males) with alcoholic liver disease.

The patients with acute hepatitis underwent liver biopsy when their serum aminotransferase levels had completely or nearly normalized. The patients with chronic hepatitis were observed for more than 1 year because of elevated levels of the serum aminotransferase. HCCs were biopsied incidentally or under echographic guidance.

HCV infection was diagnosed by enzyme-linked immunosorbent assay (second generation, Dainabot, Japan) and serum HCV RNA by reverse transcription followed by polymerase chain reaction of the 5′-noncoding region [12]. Hepatitis B was diagnosed by radioimmunoassay (Dainabot) for hepatitis B surface antigen (HBsAg). The patients with positive HBsAg were negative for anti-HCV, and those with alcoholic liver disease were negative for both anti-HCV and HBsAg.

Two kinds of antibody against the HCV core polypeptides (recombinant and synthetic) were prepared. HCV cDNA spanning amino acids 1–120 of the core gene was amplified by the polymerase chain reaction after reverse transcription using sera from Japanese patients with chronic HCV infection. The cDNA was inserted into the *Eco*R1 site of plasmid pUEX2. The fusion protein with

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beta-galactosidase was purified and inoculated into rabbits. The other antigen was a synthetic peptide spanning amino acids 1–28 of the core gene, which was also inoculated into rabbits. The polyclonal antisera obtained were purified to IgG for subsequent immunostaining. The expressed protein is used commercially in a diagnostic kit for detection of serum anti-HCV (JCC; Chemo-Sero-Therapeutic Research Institute, Japan).

For immunocytochemistry the specimens were fixed in 10% buffered formalin and embedded in paraffin. Four micrometre-thick sections were processed for indirect immunoperoxidase staining. Deparaffinized sections were reacted with the first antibody, anti-HCV at a dilution of 1: 500 at 4° C overnight, and then with the second antibody, goat anti-rabbit IgG conjugated with horseradish peroxidase (Medical and Biological Laboratories, Japan) at a dilution of 1: 500 at 37° C for 1 h. The reaction products were visualized by treatment with 3,3′-diaminobenzidine tetrahydrochloride, and the sections were briefly counterstained with haematoxylin. After each step, the sections were rinsed with phosphate-buffered saline.

For blocking control, the first antibody was absorbed beforehand with the expressed antigen (42.5 μ g antigen in 1 ml of 1: 500-diluted antibody) at 37° C for 1 h, followed by clarification by centrifugation. The second antibody was applied to sections after incubation with the normal rabbit serum or elimination of the first antibody reaction.

## Results

The HCV core antigen was positively stained in 5 (50%) of 10 cases of acute hepatitis C, 28 (70%) of 40 cases of chronic hepatitis C, 6 (75%) of 8 cases of HCV-related liver cirrhosis, and 4 (67%) of 6 cases of HCV-related HCC. All cases of acute and chronic hepatitis B and alcoholic liver disease were negative for HCV core antigen. The two kinds of antibodies yielded the same staining results. Control staining after absorption with the antigen and elimination of the first antibody reaction abolished the staining of the core antigen. Therefore the immunostaining was confirmed to be specific.

The core antigen was stained within the cytoplasm of hepatocytes and showed a finely granular distribution; the antigen was localized diffusely (Fig. 1a), perisinusoidally (Fig. 1b), or focally (Fig. 1c) in the cytoplasm with often intermediate staining patterns. The hepatocellular nuclei were negative, and it was unclear whether or not the cell membrane was stained. The percentage of the positive core antigen staining among the hepatocytes was roughly 80–100% in acute hepatitis, 20–90% in chronic hepatitis and 10–60% in cirrhosis. The HCC cells showed 30–50% positivity.

The staining was homogeneous or heterogeneous; in the former case, the core showed a uniform staining intensity and pattern throughout the whole area of the biopsy, whereas in the latter the staining varied in pattern and intensity from area to area. In the heterogeneous staining pattern, negative areas and positive areas were admixed and the staining intensity was variable among hepatocytes in the same area (Fig. 2). This heterogeneous staining pattern tended to be more conspicuous in cases of more advanced liver disease.

Necroinflammation characterized by hepatocellular necrosis associated with lymphocytic infiltration did not always coincide in strength and location with the the distribution of the HCV core antigen. Hepatocytes with intense core infection did not show any conspicuous degenerative change.

In HCCs staining was focally or diffusely positive in the carcinoma cell cytoplasm in a patchy or zonal distribution. The nuclei were negative (Fig. 3). The non-carcinomatous cirrhotic portion always showed positive staining when the HCC was positive. There was no correlation between positive HCV staining and the degree of differentiation or pattern of the HCCs.

Proliferated bile ductular cells occasionally showed positive staining (Fig. 4). The other cellular elements in-

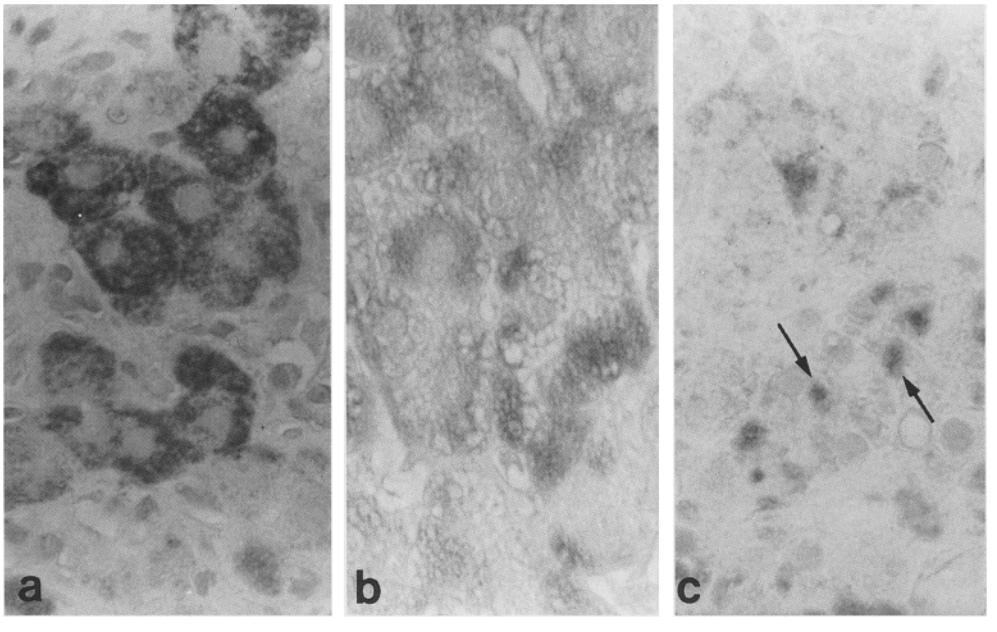


**Fig. 1a–c** Positive immunostaining of the hepatitis C virus (HCV) core antigen within the cytoplasm of hepatocytes (weakly counterstained with haematoxylin, × 400). **a** Staining is diffuse (diffuse type). **b** Staining is stronger towards the sinusoid (perisinusoidal type). **c** Staining is focal (*arrows*; focal type)

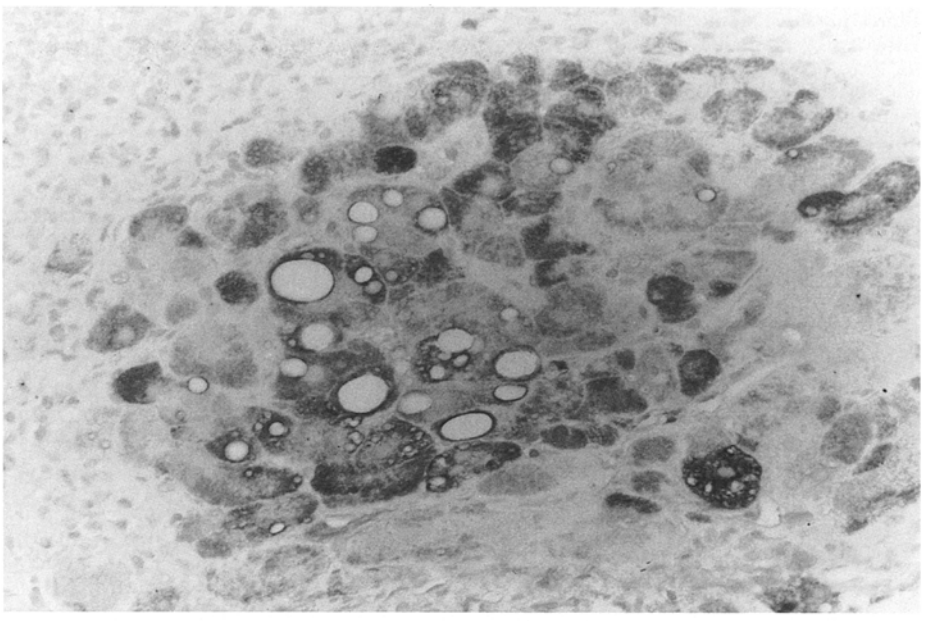

**Fig. 2** Heterogeneous immunostaining pattern of the HCV core antigen. Staining intensity is variable among hepatocytes (weakly counterstained with haematoxylin, × 200)

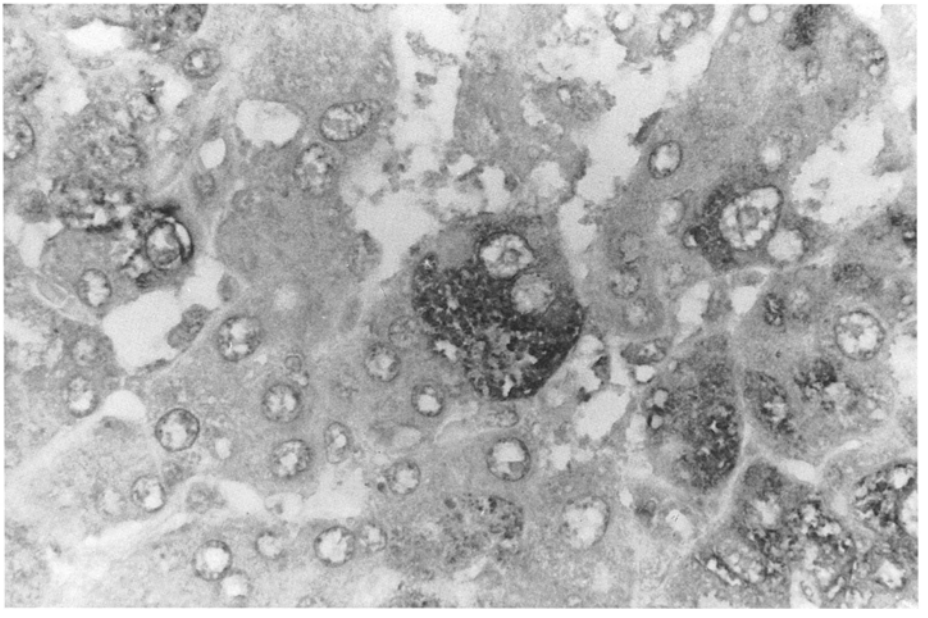

**Fig. 3** Immunostaining of the HCV core antigen in hepatocellular carcinoma. The carcinoma cells are stained in a diffuse or focal pattern within the cytoplasm (counterstained with haematoxylin, × 400)

cluding interlobular bile duct cells, Kupffer cells, endothelial cells, lymphocytes and macrophages were negative.

## Discussion

Among the non-carcinomatous cases positive for serum anti-HCV, 67% showed positive staining in needle biopsy samples. HCV might have been absent or only weakly replicated in the cases showing negative staining; it is also possible that the liver biopsy samples did not contain areas expressing the core antigen in spite of infection with HCV. Formalin-fixation and paraffin-embedding may diminish antigenicity. It is also possible that the core sequence may differ from that used for immunization, although the core gene has been shown to be highly conserved among various HCV isolates [6]. The two kinds of antibodies gave the same staining results; the antibody against the recombinant protein did not stain beta-galactosidase in the present investigation.

A slightly higher positive rate (86%) of immunostaining was recently reported from another laboratory [4] using liver frozen sections with chronic hepatitis C. According to this report, some cases were revealed to be negatively stained in spite of the presence of HCV RNA in sera, as in the present investigation.

The present results of HCV core staining were essentially identical to those in the literature [5, 9]. However, the present approach allowed more detailed histological

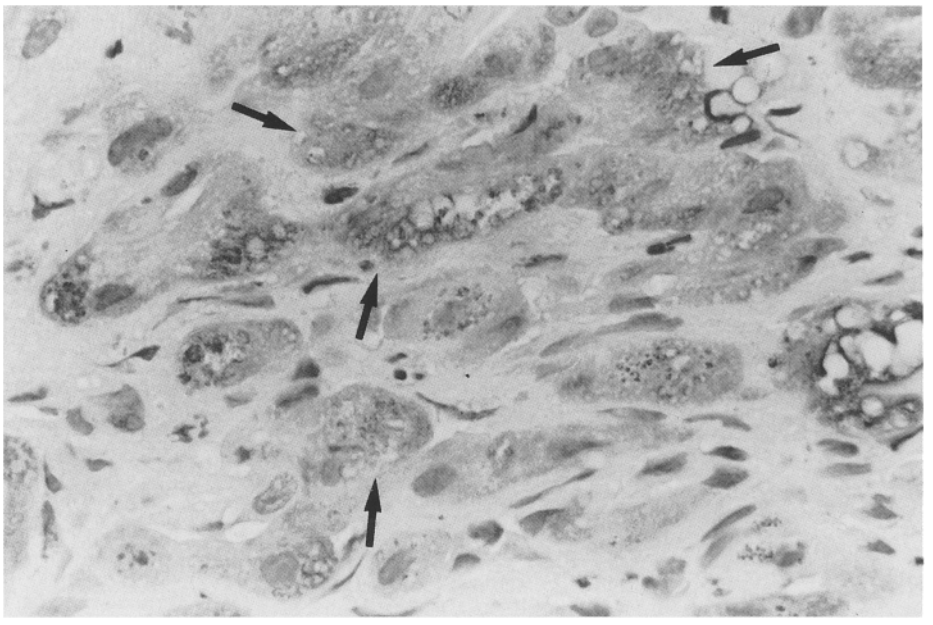

**Fig. 4** Immunostaining of the HCV core antigen in proliferated bile ductular cells (*arrows*; counterstained with haematoxylin, × 400)

observation. Immunostaining of the HCV core antigen was somewhat similar to that of HBsAg, being diffuse, perisinusoidal and focal in the cytoplasm and negative in the nucleus [13]. However, HCV core antigen lacked the distinct membrane or inclusion-type pattern seen in the latter. Heterogeneous staining was also seen for HBsAg and the heterogeneity may reflect irregular infection and replication of the HCV due to irregular regeneration preceded by irregular necroinflammation in the parenchyma, as seen in cases of HBsAg [13].

Proliferated bile ductules occasionally showed positive staining; this phenomenon can be understood when it is considered that these ductules originate from hepatic cords [15]. The absence of staining of other cellular elements in the non-carcinomatous liver strongly suggests that hepatocytes are the only cells infected with HCV, at least in the liver. HCV does not seem to infect lymphocytes, which frequently form aggregates or follicles in the portal tract [16].

The significance of HCV infection in HCC cells remains to be determed. It is uncertain whether the HCV infects HCC cells before or after carcinogenesis. It is probable that some HCV-derived proteins play a transactivating role in hepatocarcinogenesis, although such genes have not yet been specified [3, 8].

Hepatocellular necrosis in hepatitis C is considered to be immune-mediated. Some HCV-related antigens are considered to be immune targets for the sensitized cytotoxic T cells of the hosts. Since necroinflammation of the liver parenchyma does not always correspond to the location of the HCV core antigen, the core may not be the immune target; rather the envelope antigen could be a candidate [17]. The absence of degenerative changes in HCV-infected hepatocytes strongly supports the hypothesis that hepatocellular necrosis is immune-mediated in hepatitis C.

The successful immunostaining of HCV in formalin-fixed, paraffin-embedded tissue has enabled us to observe the localization of HCV in the liver tissue easily and precisely. This method will help to provide clues for clarifying a number of aspects of hepatitis C that have remained obscure, including the mechanism of HCV replication in the liver, the frequent chronicity of acute disease and the precise mechanism of hepatocellular necrosis and disease progression. Definite prediction of interferon efficacy before treatment can be attempted, interference of hepatitis B virus and HCV investigated, and the mechanism of hepatocarcinogenesis examined.

**Acknowledgements** The authors are grateful to the Chemo-Sero-Therapeutic Research Institute for providing the antigen and antisera.

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