Immunohistochemical, Molecular, and Pathomorphological Study of Liver Biopsy Specimens during Chronic Hepatitis C

G. I. Nepomnyashchikh, S. V. Aidagulova, D. L. Nepomnyashchikh, N. P. Tolokonskaya, Yu. Yu. Karavaeva, E. G. Sakharova, G. A. Mezentseva, and E. V. Batemirova

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We carried out a comparative study of replication markers of hepatitis C virus (HCV RNA) in some biological substrates and NS3-antigen in liver biopsy specimens. It was found that 82% liver specimens contained RNA HCV, in 44% cases HCV RNA was present in the serum, mononuclear blood cells, and liver. The presence of NS3-antigen in hepatocytes can be considered as a structural marker of HCV replication, which is confirmed by positive correlation with the results of PCR for viral RNA in tissue specimens. In most cases the presence of HCV replication markers did not correlate with activity of the infectious process assessed by biochemical and histological tests.

Key Words: *chronic HCV-infection; liver biopsy; polymerase chain reaction; immunohistochemistry*

The most important peculiarity of hepatitis C virus (HCV) is its long-term persistence in the host organism [5]. Despite the development of virus-specific immune response, it does not eliminate the virus in most patients and does not prevent reinfection [1]. The use of highly specific modifications of PCR and *in situ* hybridization revealed replication of HCV in many organs and tissues [11]. Moreover, genotyping showed the existence of independently replicating viral populations in individual host organism [8].

Immunohistochemical assay of HBcAg in the liver during chronic hepatitis B virus (HBV) infection revealed a positive correlation between serum level of DNA HBV and expression of HBcAg in hepatocyte nuclei. Hence, immunodetected HBV epitope can serve as a marker of HBV replication [7]. Similar relationships for HCV were not studied. The development of mouse monoclonal antibodies to recombinant NS3 protein of HCV made it possible to demonstrate virus-specific staining of hepatocyte cytoplasm during acute HCV infection and in immunosuppressive patients with chronic HCV infection. The biological role of nonstructural NS3 protein remains unknown, although some data indicate that Core, NS3, and NS5A proteins promote cell growth via modulation of cell cycle regulator genes. For example, NS5A protein interacts with common transcription factors [10]; however there are no data on integration of HCV into the genome of infected cell.

Our aim was to study the molecular and biological markers of chronic HCV infection and their correlations with pathomorphological and immunohistochemical parameters of liver biopsy specimens.

MATERIALS AND METHODS

Thirty-six patients (31 men and 5 women aged 17-54 years) with chronic HCV infection were examined (of them 24 patients aged 17-30 years). Complex examination included *1*) biochemical tests (including ala-

Laboratory of Ultrastructure Basis of Pathology, Laboratory of Clinical and Morphological Basis of Infectious Pathology, Laboratory of Clinical Morphology, Institute of Regional Pathology and Pathomorphology, Siberian Division of the Russian Academy of Medical Sciences, Novosibirsk. ***Address for correspondence:*** pathol@cyber.ma.nsc.ru. Nepomnyashchikh G. I.

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nine and aspartate aminotransferase activities); *2*) serological tests, *i.e.* immunoenzyme assay of viral markers of hepatitis A (anti-HAV-IgM), B (HBsAg, HBeAg, HBc-IgM, HBc-IgG, HBsAb, HBeAb), C (anti-HCV total antibodies and those to Core- and NS-antigens), and D (anti-HDV), and *3*) pathomorphological examination of liver biopsy specimens. Reverse transcription PCR was used for detection of HCV RNA and HBV DNA in the serum, mononuclear blood cells, urine, saliva, and liver tissue.

Transcutaneous liver biopsy was performed under local anesthesia with disposable biopsy needles (Braun). The length and diameter of the punctate were 1.0-3.5 cm and 2-3 mm, respectively. Paraffin sections for light microscopy were stained with hematoxylin and eosin followed by Perls' reaction. They were also stained by the method of van Gieson method combined with Weigert resorcin-fuchsin staining and periodic acid-Schiff reaction. The severity of hepatitis and liver fibrosis were evaluated using a Metavir system [6].

For electron microscopy and histochemical analysis liver samples were fixed in 4% paraformaldehyde on phosphate buffer 0.1 M (pH 7.2-7.4). Semithin sections were stained with azure II and Schiff reagent. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined under a JEM-1010 electron microscope.

Immunohistochemical study (detection of HCV NS3 antigen) was carried out on paraffin sections (5 μ) placed on adhesive-treated slides. Two-stage indirect immunoperoxidase method with streptavidin-biotin visualization was used. Mouse monoclonal IgG2b antibodies against recombinant NS3 protein (NS3-HCV, NovoCastra Laboratories Ltd.) diluted 1:25 and universal biotinylated antibodies were used as primary and secondary antibodies, respectively. Incubation with primary and secondary antibodies and the reaction with streptavidin-peroxidase complex were conducted at 37°C. NS3-antigen was unmasked by boiling in retrivagen A (pH 6.5). Endogenous peroxidase activity in hepatocytes was inhibited by 0.3% H_2O_2 on phosphate buffer. After visualization of the immune reaction products with chromogen (diaminobenzidine), the sections were poststained with Meier hematoxylin and placed in tap water for 10 min. The sections not treated with primary antibodies were used as the negative control.

RESULTS

HCV RNA, the molecular-biological marker of HCV replication, was found in most patients in liver tissue (82% specimens), serum (77%), mononuclear blood cells (70%), saliva samples (22%), and urine (11%). In 44% cases (16 of 36 patients) HCV RNA was simultaneously detected in three substrates: serum, mononuclear blood cells, and liver tissue. In 2 patients it was found in all examined specimens including saliva and urine. These data confirm replication of HCV in various organs and demonstrate the presence of viral RNA in various biological substrates, which is important for long-term persistence of the virus in host organism.

It is noteworthy that in 11% cases HCV RNA was detected in the liver, but was absent in the serum and blood mononuclear cells. Seronegativity can be caused by viral interference, *i.e.* suppression of HCV replication during concurrent hepatitis A or B [13]. Thus, detection of HCV replication marker in liver biopsy specimens allows rapid and accurate diagnosis of hepatitis C in anti-HCV-positive patients that are seronegative in respect to HCV RNA (60% cases [9]).

One of the leading pathognomic pathomorphological phenomena of HCV-infection was diffuse polymorphic lipid infiltration of the hepatocyte cytoplasm (87% cases). It was concluded that the phase of HCV replication (revealed by PCR) is characterized by small-vesicular submembrane lipid infiltration, which often looked like a pearl necklace (Fig. 1, *a*). Lipid infiltration of hepatocytes probably reflects emergency repair reaction of the cell to the direct cytopathic effect of the virus penetrated into the cell via endocytosis. The relationship between viral proteins and expression of phenotypic alterations was studied on transgenic mice strains with HCV-core-antigen. It was found that this antigen directly participates in the development of hepatic steatosis [12].

Electron microscopy of biopsy specimens (Fig. 1, *b*) showed that small lipid inclusions are regularly located near the cytolemma of hepatocytes without degeneration signs (depletion of cytoplasmic matrix caused by inhibition of synthetic processes) [3,4]. This confirms the assumption that involution degeneration is a morphological manifestation of antiviral (preventive) strategy of hepatocytes [2].

Immunohistochemical assay of HCV NS3 antigen revealed positive hepatocytes in various parts of the lobule; in some cases, NS3 was detected in the majority of parenchymatous cells (Fig. 2, *a*, *b*), but the most pronounced reaction was found in zones of periportal lymphoid infiltration (Fig. 2, *c*, *d*). In most cases, detection of antibodies to NS3 in the serum was accompanied by expression of NS3 in hepatocytes. Intensive or moderate immunohistochemical reaction with NS3 antigen in liver biopsy specimens was often found in individuals with normal amino transferase activity.

The presence of HCV RNA in liver tissue correlated with immunohistochemical detection of NS3 antigen. Thus, this antigen can be considered as a struc-

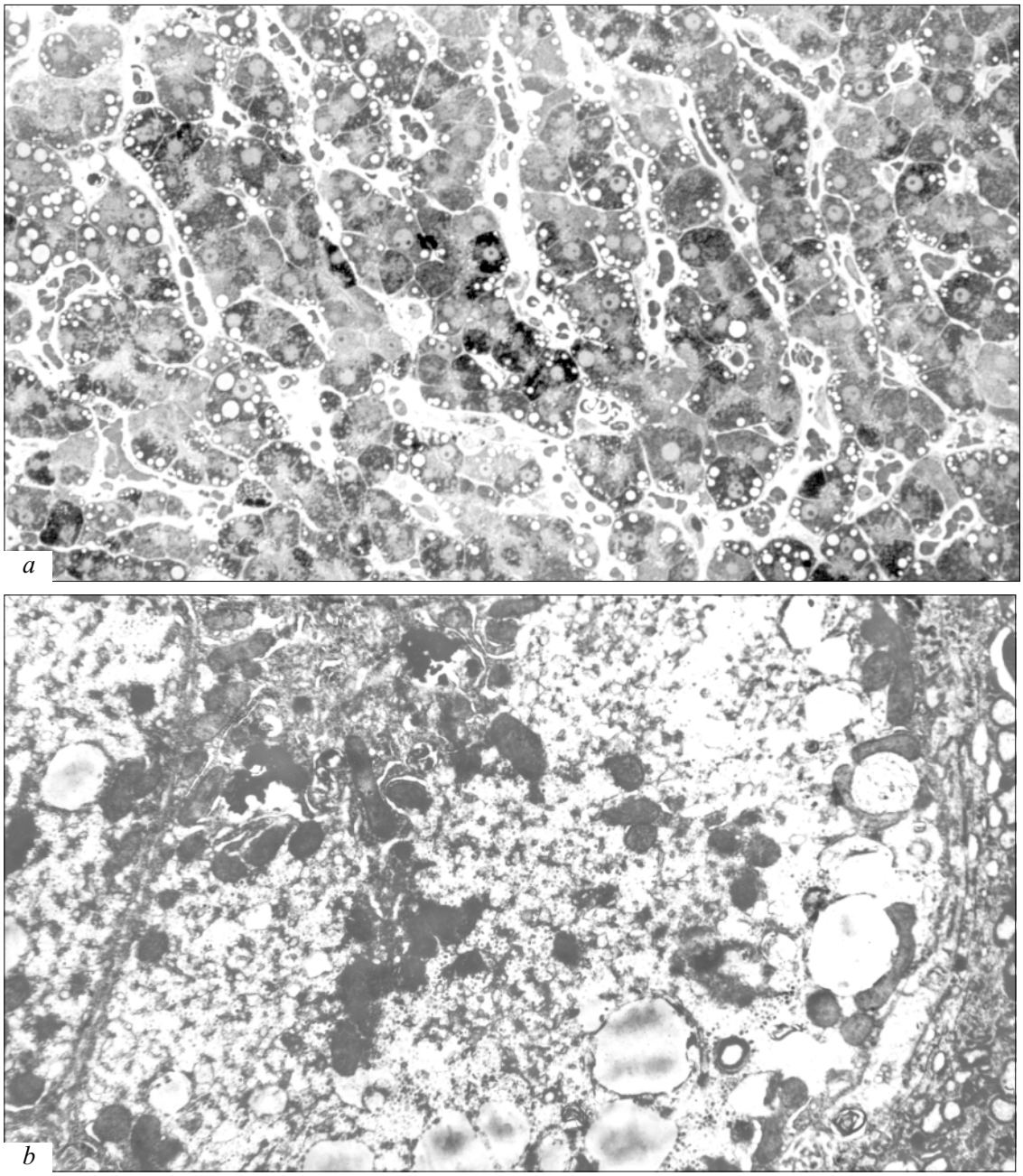

Fig. 1. Chronic hepatitis C. Liver biopsy specimen, ×100 (*a*), ×8000 (*b*). *a*) diffuse small vesicular submembrane lipid infiltration of hepatocytes. Semithin section stained with Schiff reagent and azure; *b*) a fragment of hepatocyte cytoplasm with a cluster of lipid droplets near the cytolemma.

tural marker of HCV replication. The complex of markers (HCV RNA and viral epitopes detected by immunohistochemical technique) notably increases the diagnostic value of liver biopsy.

In most cases, examination of liver biopsy specimens revealed only minor HCV activity, the absence of necrotic and necrobiotic changes in parenchyma cells, moderate mononuclear infiltration of the stroma). Only in 4 bioptates necrobiosis and pronounced mononuclear (lymphoid predominantly) infiltration of the stroma and parenchyma were found, which correlated with the increase in biochemical indices of cytolysis.

It should be emphasized that according to biochemical and histological data, the presence of HCV replication markers (HCV RNA and NS3Ag in hepatic tissue) did not correlate in most cases with the severity of the infectious process. The dynamics of the infectious process is determined by a complex of various factors: the level of viremia, heterogeneity and vari-

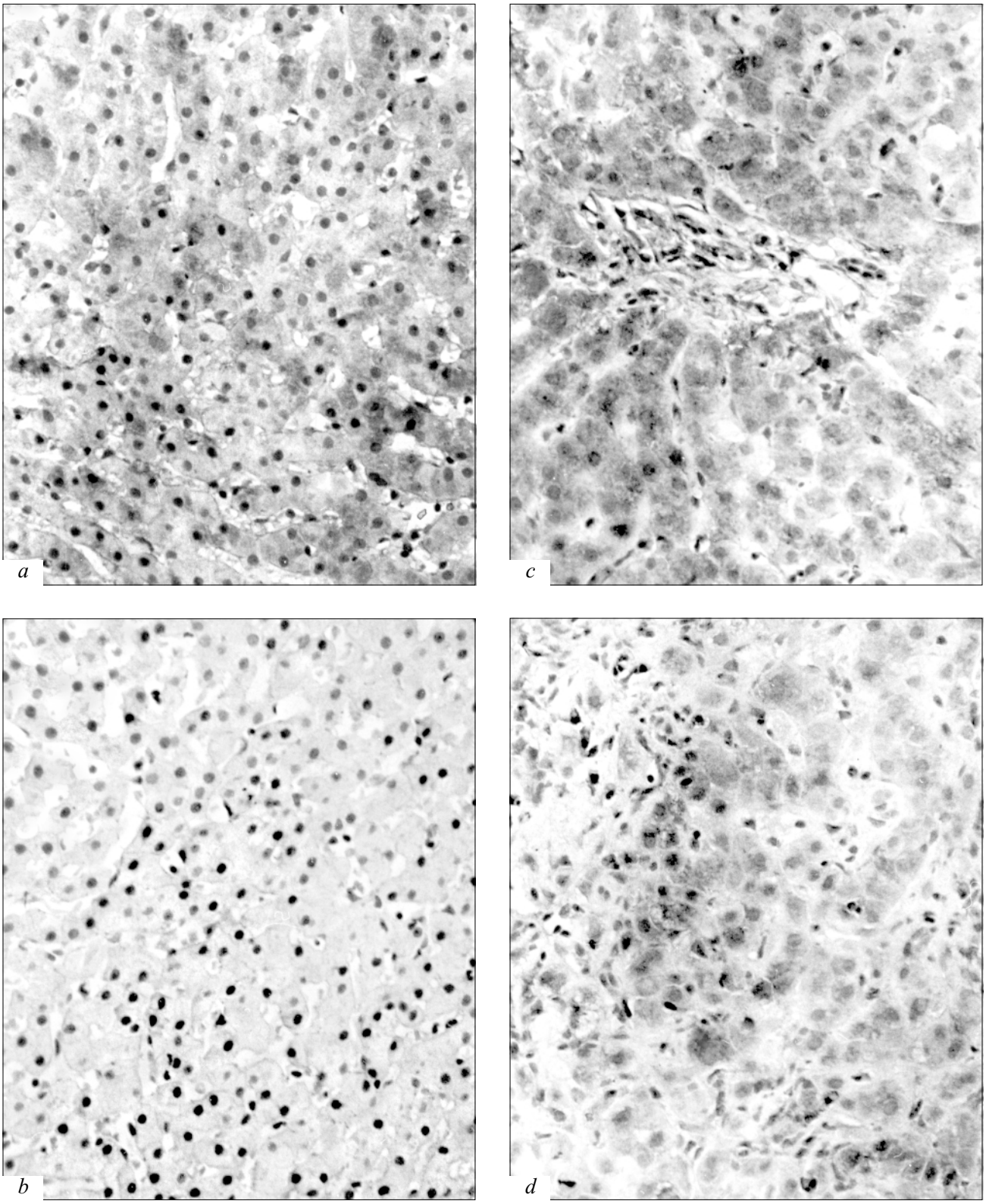

Fig. 2. Liver biopsy specimen from a patient with chronic HCV-infection. Paraffin sections, indirect immunoperoxidase method, staining with diaminobenzidine and hematoxylin, ×300. *a*) NS3-positive hepatocytes in the centrolobular zone; *b*) control section without primary antibodies: reaction is absent; *c*) intensive expression of NS3-antigen in periportal hepatocytes; *d*) a cluster of NS3-positive hepatocytes in the zone of inflammatory cell infiltration.

ability of individual virus population, the state of cell and humoral immunity, mobilization of antiviral strategy of hepatocytes, and weak cytopathic potency and long-term persistence of HCV in the organism.

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