Molecular, Genetic, and Morphological Markers during Persistence of RNA-Containing Hepatitis C Virus in the Body

N. P. Tolokonskaya, G. I. Nepomnyashchikh, V. I. Konenkov, and S. V. Netesov

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A comparison of 5'-UTR structures in Siberian isolates of hepatitis C virus showed that the majority of genotyped samples had 1b genotype. A correlation was found between HLA antigens and their stable complexes and clinical manifestations of hepatitis C. We revealed immunogenetic criteria of organism's resistance or susceptibility to hepatitis C virus. The presence of hepatitis C virus RNA in the serum determined by the reverse transcription polymerase chain reaction was accompanied by the appearance of pathomorphological signs characteristic of liver tissue infection, but did not correspond to the severity of fibrosis.

Key Words: *hepatitis C; polymerase chain reaction; genotyping; HLA antigens; liver biopsy; hepatocytes*

Considerable problems in clinical diagnostics of hepatitis C are due to natural variability of RNA-containing hepatitis C virus (HCV) and its antigens the existence of, numerous molecular and genetic isolates in Russia, and asymptomatic or latent course of the disease [13]. HCV is transmitted by artificial routes; the population at high risk for hepatitis C include intravenous-drug abusers. A considerable number of HCV carriers are drug abusers, which promotes virus passage and modulates phenotypic manifestations of this disease [3].

Polymerase chain reaction (PCR) is a highly sensitive method. It allows identification of hepatitis agent in the serum, liver tissue, and peripheral blood mononuclear cells [10]. This technique has considerable advantages in detecting and monitoring viral hepatitis C (VHC), in particular, in the absence of antibodies against HCV antigens. Apart from PCR, molecular, genetic, serologic, immunologic, and pathomorphological methods are used to study viral hepatitides [3,4,6].

Here we studied HCV infection markers using molecular, biological, immunogenetic, and pathomorphological methods.

MATERIALS AND METHODS

We examined 90 patients (majority of them below 30 years) with VHC; 38 patients were opioid abusers. Serum markers of hepatitides A (anti-HAV IgM), B (HBsAg, HBeAg, anti-HBc IgM, anti-HBs, and anti-HBe), C (total anti-HCV, anticore, and anti-NS-HCV), and D (anti-HDV) were detected by enzyme immunoassay.

HCV RNA in blood samples was detected by reverse transcription PCR (RT-PCR) [14]. Sequencing of the amplification products was performed by the method of Sanger using Tag DNA polymerases [11].

HCV genotyping was performed by comparing 5'-UTR sequences and NS5B gene regions in HCV isolates with Genbank Rel. 108 (8/98), EMBL, and DDBJ data. Multiple alignment of nucleotide and estimated

Department of Infectious Diseases, Novosibirsk State Medical Academy; Laboratory of Ultrastructural Basics of Pathology, Institute of Regional Pathology and Pathomorphology, Siberian Division of the Russian Academy of Medical Sciences; Laboratory of Clinical Immunogenetics, Institute of Clinical Immunology, Siberian Division of the Russian Academy of Medical Sciences; Laboratory of Molecular Biology of RNA-Containing Viruses, State Research Center Vektor, Novosibirsk. ***Address for correspondence:*** pathol@cyber.ma.nsc.ru. Tolokonskaya N. P.

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TABLE 1. Distribution of HLA Antigens and Haplotypes during VHC (%)

HLA	Control (*n*=680)	Patients (*n*=68)	DC	RR
A10	15.74	32.35*	3.13	2.56
DR4	22.41	0*	-14.90	-39.82
DR5	41.91	58.82**	1.47	1.98
DR2-DR5	8.71	20.59**	3.73	2.72
DR5-DR7	6.22	19.12**	4.87	3.56
A2-B0	17.94	0*	-13.90	-30.05
B27-DR1	0.49	10.29*	13.26	23.52

Note. Here and in Tables 2 and 3: *p<0.001, **p<0.01, and ***p<0.05 compared to the control. DC: diagnostic coefficient.

amino acid sequences was carried out using PHYLIP V3.57c software [7].

HLA system antigens were determined by immunogenetic assay of peripheral blood lymphocytes based on the complement-dependent cytotoxicity using typing monospecific antiserum kits (Institute of Hematology and Blood Transfusion, St.-Petersburg). The relative risk (RR) and the incidence of HLA antigens, genes, phenotypes, and haplotypes in various HLA loci were estimated to reveal the distribution of histocompatibility complex class I and II antigens. Significant differences between the incidence of HLA markers and HLA associates in various groups were evaluated by precise Fisher's test and χ^2 test.

Transcutaneous biopsy of the liver was performed in 63 patients using Braun puncture needles. A total of 77 samples were examined. For light microscopy, liver samples were fixed in 10% neutral formalin. Paraffin slices were stained with hematoxylin and eosin and by the van Gieson's method combined with the Perls reaction. Elastic fibers were stained with Weigert's resorcin-fuchsin and Schiff's reagent.

Samples for electron microscopy were fixed in 4% paraformaldehyde, postfixed with OsO_4, and treated by routine methods [3]. Semithin slices were stained with azure II and Schiff's reagent. Ultrathin slices were contrasted with uranyl acetate and lead citrate and examined under a JEM 1010 electron microscope.

RESULTS

Total anti-HCV were detected in all blood samples. However, anticore and anti-NS-HCV were simultaneously found only in 56 of 90 samples. The presence of HCV RNA in 60 samples was confirmed by RT-PCR. The HCV genotype was determined in 46 samples. In 78% samples with estimated HCV genotype, genotype 1b was dominant. However, previous studies of HCV showed that the epidemiology of this infection and the genotype structure of infectious agents undergo considerable and rapid changes.

The etiological factor plays an important role in VHC, but it should be emphasized that genetically determined peculiarities of the protective systems determine universal reactions (including negative response) to damaging factors. Previous studies demonstrated an interrelation between some genetic markers and the susceptibility to autoimmune hepatitides induced by various etiological factors [1]. The data suggest that it is necessary to analyze the state of a patient with HCV markers, but not VHC characteristics.

Immunogenetic assays of European population in West Siberia showed high incidence of HLA-A10 and HLA-DR5 antigens and DR2-DR5, DR5-DR7, and DR1-B27 combinations and the absence of HLA-DR4

TABLE 2. Distribution of HLA Antigens and Haplotypes during Acute VHC (%)

HLA	Control (*n*=680)	Patients (*n*=20)	DC	RR
DR7	26.14	60.00***	3.61	4.24
B15-B18	0.15	10.00***	18.33	75.44
B27-B35	0.15	10.00***	18.33	75.44

TABLE 3. Distribution of HLA Antigens and Haplotypes during Chronic VHC (%)

HLA	Control (*n*=680)	Patients (*n*=48)	DC	RR
A10	15.74	33.33***	3.26	2.68
DR1	21.58	41.67**	2.86	2.60
DR4	22.41	0*	-13.40	-28.19
A9-A10	2.65	12.50**	6.74	5.25
DR2-DR5	8.71	25.00**	4.58	3.49
A2-B0	17.94	0*	-12.40	-21.28
A2-B41	0.15	8.33*	17.53	61.73
A9-DR5	11.65	33.33*	4.57	3.79
B27-DR1	0.49	12.50*	14.11	29.29

antigens in patients with VHC (Table 1). These results indicate that the resistance or susceptibility to VHC is associated with genes of the major histocompatibility complex.

The relationship between HLA antigens and clinical manifestations of the disease showed high incidence of 3 immunogenetic HLA markers, DR7, B15-B18, and B27-B35, during acute VHC (Table 2). Chronic VHC is accompanied by changes in the distribution of 8 markers, including low incidence of HLA-DR4 antigens and high incidence of HLA-A10, HLA-DR1, A9-A10, DR2-DR5, A9-DR5, and B27-DR1 (Table 3).

Studies of the interrelation between immunogenetic markers and the predisposition to drug abuse in patients with viral hepatitis and history of drug addiction revealed low incidence of the HLA-A11 specificity (RR=-2.12) and high incidence of HLA-B7 (RR= 2.87), HLA-B40 (RR=11.26), and their combinations.

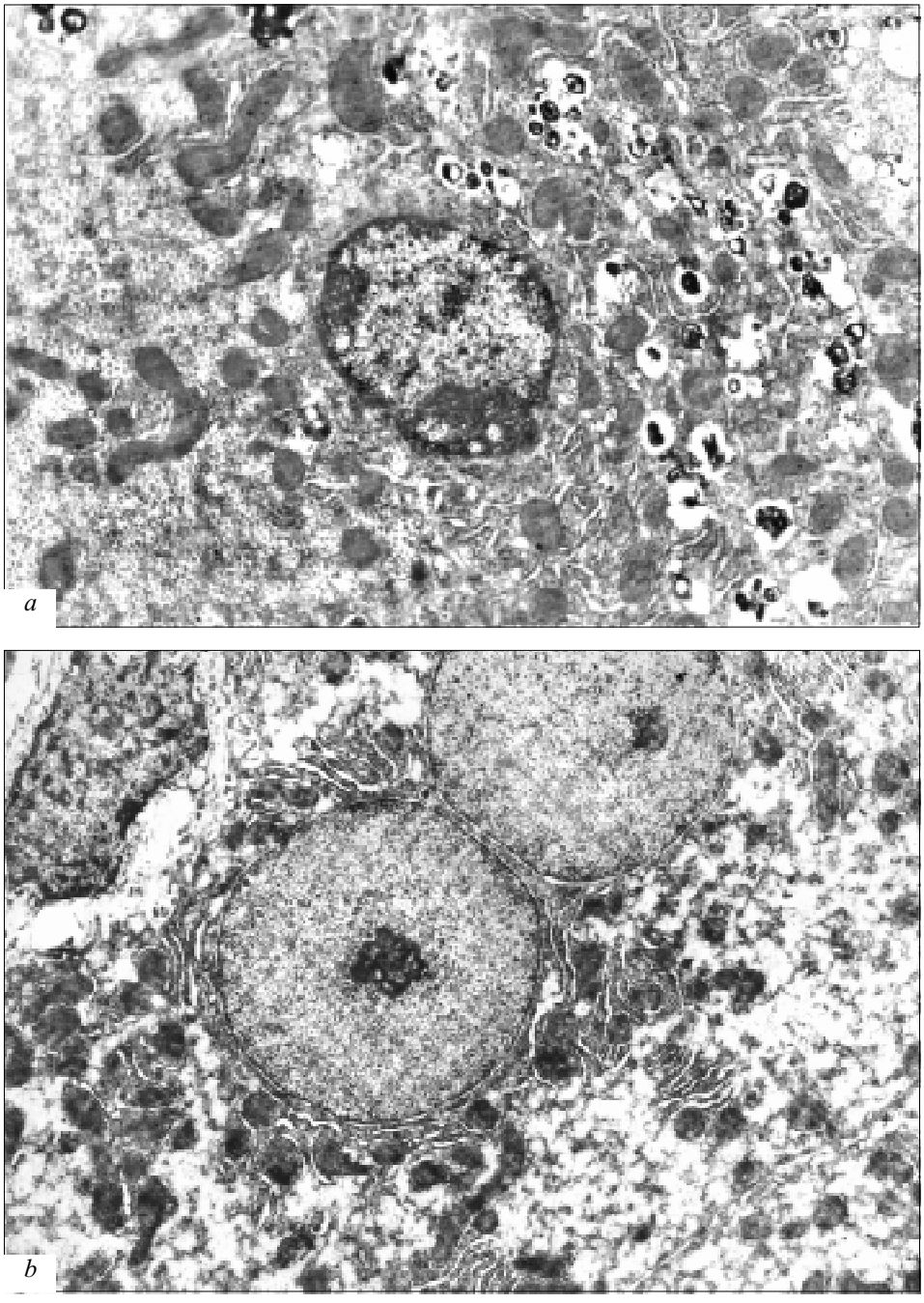

Fig. 1. Ultrastructural changes in hepatocytes during chronic hepatitis C: numerous residual bodies at the sinusoidal pole of hepatocytes (×4000, *a*); partial depletion of the cytoplasmic matrix, preserved nuclear compartment, and perinuclear accumulation of mitochondria and profiles of granular cytoplasmic reticulum (×4500, *b*).

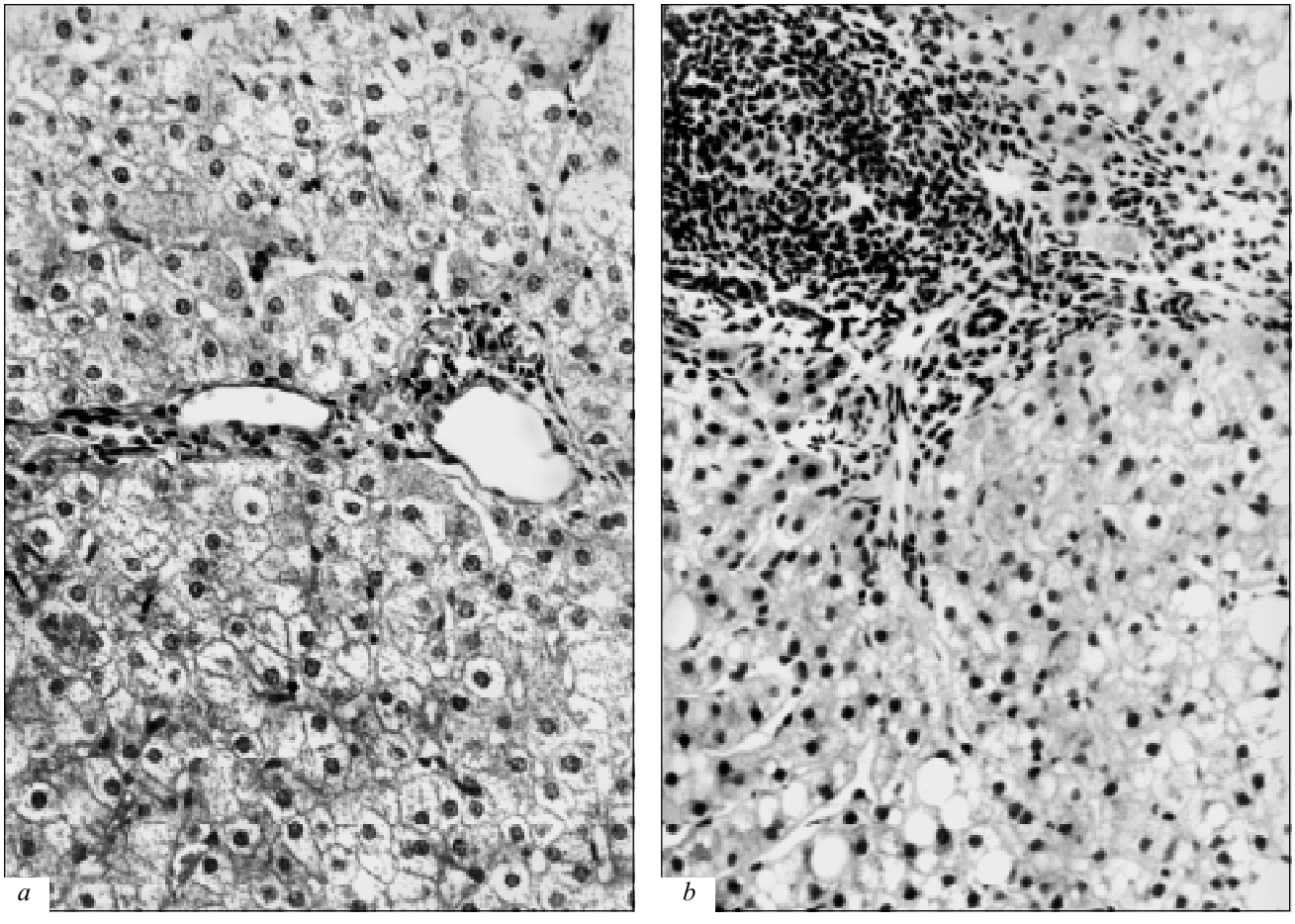

Fig. 2. Light microscopy if liver bioptates during chronic hepatitis C: atrophy of hepatocytes and moderate cell infiltration of portal tracts (PCR^-, Van-Gieson's staining, ×250, *a*); lipid infiltration of hepatocytes, pronounced cell infiltration in parenchyma and lymphoid follicles of portal tract (PCR^+, hematoxylin and eosin staining, ×220, *b*).

Studies of liver bioptates from patients with VHC revealed various structural changes, including lipid infiltration of hepatocytes, lymphoid follicles, and damages to bile ducts [5]. We also observed predominance of cell involution atrophy characterized by reduction of cytoplasmic organelles and depletion of cytoplasmic matrix. These findings give a new notion of antiviral reactions in parenchymal liver cells during VHC, which includes inhibition of biosynthetic processes in hepatocytes, blockade of HCV replication, degradation of the cytoplasmic compartment and exocytosis (sanation), and elimination of viral particles and residual bodies (Fig. 1, *a*). Preserved hepatocyte nuclei, partial degradation of the cytoplasmic compartment, and the presence of small mitochondria and profiles of the granular cytoplasmic reticulum in the perinuclear zone attest to reversibility of these changes (Fig. 1, *b*).

Chronic VHC was accompanied by minimal or moderate infectious processes (79% samples), insignificant fibrosis (74% samples), and perisinusoidal and pericentral scleroses (50% samples). Necrotic changes in parenchymal cells were least pronounced. Lymphoid aggregates and follicles appeared in the stroma (50% samples).

We performed a comparative pathomorphological assay of liver bioptates from patients with positive or negative RT-PCR in blood samples. Minimal or moderate pathological changes in the liver were associated with negative reaction to HCV RNA (Fig. 2, *a*), while positive RT-PCR was accompanied by more severe hepatitis and lipid infiltration of hepatocytes (stages II and III). Cell infiltration in liver parenchyma, gradual necroses, postnecrotic granulomas, and lymphoid aggregates and follicles in the portal stroma were found (Fig. 2, *b*). At the same time, we found no interrelation between the severity of fibrosis and the presence or absence of HCV replication markers. However, it should be emphasized that HCV RNA was always found in the serum of patients with stage III liver fibrosis.

A correlation between pathomorphological changes in the liver during VHC and HCV RNA content in the serum and liver tissues is poorly understood. It

was shown that the content of HCV RNA in liver tissue correlates with that in the serum [9]. However, there is no correlation between the severity of histological changes in the liver and HCV RNA contents in the serum and liver tissue in patients with chronic active hepatitis. Probably, serum content of HCV RNA does not completely correspond to HCV level in liver tissue.

In many cases, PCR does not detect HCV in liver samples from patients with antiviral antibodies in the serum [13]. At the same time, PCR reveals HCV RNA in liver tissues of patients with VHC complicated by liver cirrhosis [8].

Comparative analyses of clinical and epidemiological parameters, detection of antibodies to viral epitopes and HCV RNA in the serum, and pathomorphological assay of liver bioptates hold much promise for estimating clinical variants of VHC. Our experiments indicate that acute clinical manifestations of the disease (suspected long-term infectious process) accompanied by the appearance of antibodies against anti-NS-HCV without HCV RNA in the serum are episodes of chronic infections. These data were confirmed by microscopy of liver bioptates demonstrating signs of chronic hepatitis: portal, periportal, and perisinusoidal fibroses, characteristic changes in hepatocytes, and mononuclear infiltration of portal tracts.

Immunogenetic assay revealed a negative correlation between acute hepatitis and the presence of HLA-DR4, while chronic hepatitis was associated with the presence of HLA-DR1 antigen and its complexes. Thus, HLA antigens and their stable combinations are associated with clinical variants of VHC. This diagnostic coefficient allows us to predict possible variants of VHC even on the basis of individual immunogenetic criteria.

Studies of VHC should include clinical, serological, immunological, genetic, and pathomorphological assays, as well as analyses of changes at the molecular level. HCV can modulate properties of structural and serum proteins. These physicochemical processes comprise not only quantitative, genetic, and chemical modifications, but also conformational changes in proteins [2], which probably play an important role in the pathogenesis of persistent viral infections, including VHC.

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