Humoral Immune Response to Recombinant Viral NS3 Protein in Patients with Hepatitis C

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 139, No. 1, pp. 89-93, January, 2005
Original article submitted November 3, 2004

Full-length recombinant NS3 protein was used in a test system for detection of specific antibodies in the sera from patients with hepatitis C. Possible antigenic determinants in NS structure were predicted. It was demonstrated that serological analysis requires enzyme immunoassay with full-length NS3.

Key Words: *hepatitis C virus; NS3 protease helicase; enzyme immunoassay*

Hepatitis C virus (HCV) causes chronic hepatitis leading to cirrhosis and hepatocellular carcinoma [8,10, 14]. The immune system of the majority of convalescents after acute hepatitis C is incapable of eliminating the virus, thus allowing its long replication in hepatocytes and some other cells. The patients develop a pronounced humoral and cellular immune response to structural and nonstructural proteins of the virus and are not protected from repeated infection. The probability of chronic hepatitis C is very high. These facts indicate the absence of protective immunity after acute hepatitis C [9,14].

NS3 protein is a nonstructural HCV protein with some catalytical functions. The N-terminal domain (180 amino acids) possesses protease activity. This serine protease participates in the formation of almost all viral nonstructural proteins [1,12]. The C-terminal domain of NS3 protein possesses ATPase/helicase activity. Antibodies to the protease domain are produced more rarely (in 10% examinees) than to ATP-helicase domain [4,7].

The titers of antibodies to NS3 protein are often low in convalescents at the end of the acute phase before virus elimination. Long-term (over 2 months) circulation of anti-HCV IgM suggests chronic transformation of the process [5,11].

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NS3 protein attracted special attention as an object for creation of DNA vaccines. Preclinical studies demonstrated the possibility of effective elimination of the virus after immunization with the plasmid containing NS3 protein gene. This led to development of both humoral and cellular antiviral immune response [9].

Modern diagnosis of hepatitis C is based on the detection of antibodies to HCV (anti-HCV) and HCV RNA. The most prevalent modern diagnostic method is enzyme immunoassay (EIA). In hepatitis C antibodies are detected by EIA, because the concentrations of protein antigens are extremely low and are virtually undetectable in the serum. Diagnostic preparations containing recombinant or synthetic fragments of virus proteins are used now.

RecombiBest anti-HCV spectrum test system (Vektor-Best company) includes components based on recombinant HCV antigens corresponding to protein sites encoded by structural (Core) and nonstructural (NS3, NS4, NS5) domains of HCV genome. These components are separately immobilized on the surface of wells of a dismountable polystyrene plate [3]. NS3 belongs to the HCV 1b subtype and contains 195 to 452 amino acid residues (about half of full-length HCV NS3) [3] (Fig. 1, *a*). All virus isolates can be divided into 6 genotypes or 11 subtypes, depending on the degree of genome similarity. Moreover, the virus is present in infected patients as a collection of virions containing slightly modified, but closely related geno-

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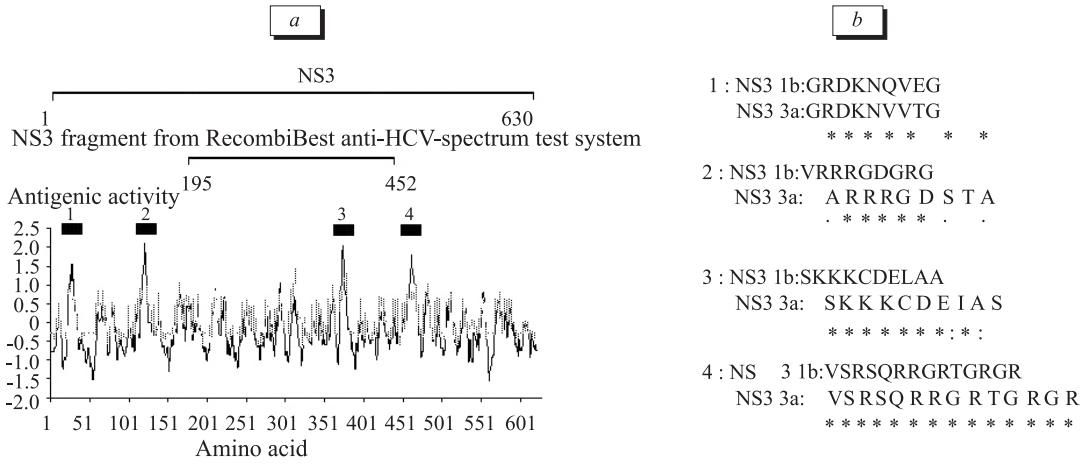

Fig. 1. Prediction of antigenic epitopes (1-4) in the structure of recombinant NS3 by Hopp and Woods' method (continuous line) and Jameson and Wolf's method (dotted line; *a*), comparison of amino acid sequences of subtypes 1b and 3a in the 4th antigenic epitope of recombinant NS3 (*b*). *Complete coincidence.

mes (quasi-species). The prevalence of different genotypes varies appreciably in different geographic regions. The 1b genotype is the most prevalent in Russia, less so are 3a, 1a, and 2a (listed in the order of decreasing incidence). The differences between the genotypes at the nucleotide level constitute 31-34%, between subtypes 20-23%, and between quasi-species 1-9%. The difference between 1b and 3a genotypes at the amino acid level is 17% [9].

Our aim was to obtain recombinant NS3 for the detection of specific antibodies in the sera of HCV patients and evaluation of the role of epitopes in the development of immune response.

We compared the immune responses to recombinant fragment (195-452) and full-length NS3 (1b subtype) in patients with different subtypes.

MATERIALS AND METHODS

Subtype 1b cDNA was used for cloning the HCV gene fragment encoding full-length NS3. Primers with sites for BgLII and XbaI restrictases in the direct and reverse primers, respectively: 5'-(T/G)TGGCGAGAT CTCGCGCC(C/T)ATGACGGCCTA-3' (direct) and 5'-CA(G/A)GTGCTCTAGACGACCTA(C/A)AGGT CAGC-3' (reverse) were constructed for PCR amplification of NS3 cDNA.

Cloning was carried out using *E. coli* M-15(rep4) strain and pQE4 plasmid (Quagen) as described previously [6]. Expression was induced using isopropyl-β-D-thiogalactoside (IPTG).

Protein from incorporated particles was purified as described previously [2]. The precipitate containing 0.4 mg recombinant NS3 was dissolved in 1 ml solution of 6 M guanidine-HCl, 50 mM Tris-HCl (pH 8.0), and centrifuged for 30 min at 13,000*g* at ambient temperature. For EIA, 1 ml denatured protein was added (dripped) to 100 ml cold carbonate-bicarbonate buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6).

NS3 protein was transferred to 96-well plates (10 μg/ml in carbonate-bicarbonate buffer, pH 9.6; 100 μl per well) and left overnight at 4°C. The plates were then washed 3 times in 200 μl phosphate saline buffer (PSB) with 0.05% Twin-20. Nonspecific binding was blocked with 3 mg/ml human serum albumin in PSB (HSA/PSB) at 37°C for 1 h, after which the sera from HCV patients diluted 1:40 in HSA (0.1%)/PSB/Twin-20 were added to the wells. Antihuman antibodies conjugated with horseradish peroxidase were added and the mixture was incubated for 1 h at 37°C. The plates were then washed 3 times with buffer, o-phenylene diamine (Sigma) solution in citrate-phosphate buffer (0.4 mg/ml o-phenylene diamine in 50 mM sodium citrate solution, 25 mM sodium phosphate solution, 0.012% hydrogen peroxide, pH 5.0) was added, and the mixture was incubated at 37°C until staining development. The reaction was stopped by adding 50 μl 50% H_2SO_4 into each well. Absorption was measured at λ=492 nm on a Multiscan spectrophotometer (LKB).

RESULTS

Bacterial clones containing plasmids with the expected fragment (according to restriction analysis) were used for the expression. Expression of recombinant NS3 in transformed *E. coli* cells was controlled by 12% sodium dodecylsulfate-PAAG electrophoresis. The maximum expression was attained 4 h after IPTG induction. The expression was much higher at 28°C than at 37°C. Recombinant protein accumulated mainly as incorporation bodies (Fig. 2).

Recombinant NS3 was used for detection of antibodies to NS3 in the sera from HCV patients (the sera

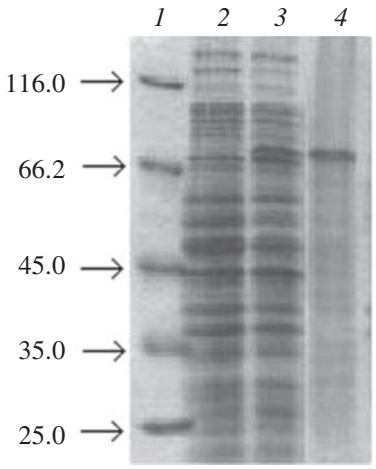

Fig. 2. Expression and purification of recombinant NS3 protein of hepatitis C virus (HCV); 12% PAAG electrophoresis with sodium dodecylsulfate. *1*) marker; *2*) lysate of initial *E. coli* M15(rep4) cells; *3*) lysate of *E. coli* transformed by recombinant NS3; *4*) purified incorporation bodies of recombinant NS3.

were received from Institute of Transplantology and Artificial Organs). Vector Best test system was used for comparison. A total of 24 sera from patients with hepatitis C were used. Antibodies to NS3 fragment were detected by the Vector Best test system in 17 of these sera (Table 1, 8-24, group 2) and no antibodies were detected in 7 sera (1-7, group 1). Positive (+Control) and negative (-Control) control samples were the same in all EIA and corresponded to those in the Vector Best test system (Fig. 3, Table 1). Full-length recombinant NS3 poorly reacted with donor sera, which could be due to the presence of admixtures not removed during purification.

We observed a pronounced reaction of recombinant NS3 with the sera from HCV patients, including those which were anti-NS3-negative in the tests with the Vector Best kits.

Antigenic epitopes in NS3 structure were predicted using the methods of Hopp and Woods and

TABLE 1. Antibodies to Different HCV Antigens Detected by Vector Best Test System in Sera from HCV Patients

Serum No.	Presence of antibodies to HCV	Core			HCV subtype
		NS3	NS4	NS5	
+Control	+	+	+	+	1b
-Control	-	-	-	-	
1	+	-	-	-	Not typed
2	+	-	+	+	2a
3	+	-	+	+	3a
4	+	-	+	+	1b
5	+	-	+	-	3a
6	+	-	-	-	Not typed
7	+	-	-	-	2a
8	+	+	+	+	3a
9	+	+	-	-	1b
10	+	+	+	+	1b
11	+	+	+	+	1b
12	+	+	-	-	1b
13	+	+	-	-	1b
14	+	+	-	-	1b
15	+	+	+	+	2a
16	+	+	+	+	1b
17	+	+	+	-	1b
18	+	+	+	+	Not typed
19	+	+	+	+	1b
20	+	+	+	+	1b
21	+	+	-	-	1b
22	+	+	+	-	1b
23	+	+	+	+	1b
24	+	+	+	+	3a

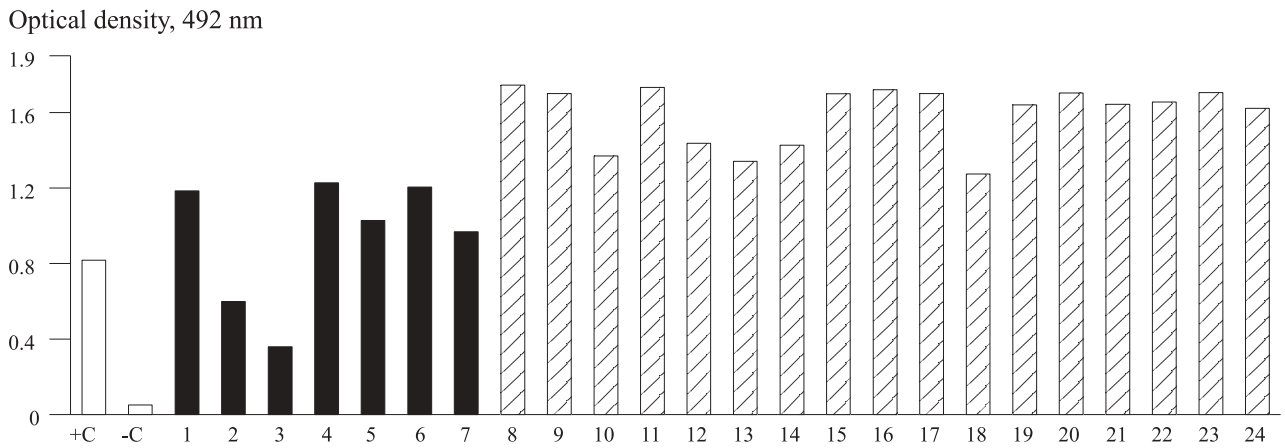

Fig. 3. Reaction of recombinant NS3 with sera of hepatitis C patients and donors in enzyme immunoassay (serum dilution 1:40). Numbers of sera in the figure correspond to serum numbers in Table 1. Light bars: control (+C, positive, and -C, negative); dark bars: +HCV, -NS3; cross-hatched bars: +HCV, +NS3.

Jameson and Wolf [16]. The method of Hopp and Woods is based on the analysis of primary protein structure and prediction of antigenic epitopes on the basis of local hydrophilia of amino acid residues. The method of Jameson and Wolf is based on analysis of availability of amino acid residues for the solvent, flexibility of the carbon skeleton, and peculiarities of secondary structure. Both methods gave virtually the same profile; 4 main antigenic epitopes can be distinguished: 23-31, 116-124, 370-379, and 456-469 amino acid residues (Fig. 1). The peptide used in the Vector Best test system includes only one of these epitopes (370-379).

The sera from hepatitis C patients used in the experiment had mainly 1b and 3a subtypes (Table 1). Site 4 is the most probable common antigen for the 3 predicted epitopes. Comparison of amino acid sequences of subtypes 1b and 3a in the 4th antigenic epitope showed complete homology of these sites (Fig. 1, *b*). Hence, this epitope determines anti-NS3 antibodies in patients with hepatitis C with subtypes 1b and 3a. Analysis of the tertiary structure of NS3 molecule [13] indicates that this epitope is a loop exposed on the molecule surface.

Antibodies to NS3 appear very rapidly after infection [9], but they cannot be identified by synthetic dodecapeptides, which indicates the conformation structure of the epitopes. A permanent low level of antibodies to NS3 is observed in chronic infection. The antibodies in this case react mainly with the C-terminal domain and recognize the peptide antigens. The use of peptides and full-length NS3 protein for antibody detection can be an important test for the diagnosis of the infection stages.

Hence, we obtained HCV full-length recombinant NS3 protein and showed the possibility of using this protein in test systems for the detection of anti-NS3 antibodies in the sera from hepatitis C patients. Further studies of this protein will explain the differences in the mechanism of immune response to viruses with different genotypes and for preparation of therapeutic vaccine [9].

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