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CHARACTERIZATION OF MONOCLONAL ANTIBODIES AND EPITOPE MAPPING OF THE NS4 PROTEIN OF HEPATITIS C VIRUS

Abstract
Recombinant DNA containing sequences of HCV NS4 protein was expressed in *Escherichia coli* cells. Six hybridoma clones producing monoclonal antibodies (MAB) to recombinant NS4 protein (rNS4), aa 1677-1756, were developed. Mapping with a panel of 33 peptides and reciprocal competitive EIA have shown that MAB obtained revealed five antigen determinants, not described earlier: MAB 3F11 and 3F12-one genotype-independent epitope of NS4A (aa 1700-1707) common for genotypes 1, 2 and 3; MAB 1D11-genotype-independent epitope (aa 1713-1728) and MAB 1D3-genotype (subtype 1b) - specific epitope of NS4B (aa 1711-1731); MAB 6B11 and C1-two conformation-dependent determinants in 5-1-1 region. These data indicate that the 5-1-1 region of NS4 protein has a complex antigenic structure and contains at least eight epitopes, including five, revealed in the present work. MAB obtained recognized native viral protein in the cytoplasm of liver cells of patients with chronic hepatitis C. The positive rates of the immunostaining for NS4 antigen using MAB 6B11, 1D11 and 3F12 were 64, 59 and 50%, respectively. It was found that 6B11 MAB to a conformation-dependent epitope much more actively interacts with native NS4 than with the recombinant protein to which MAB was developed. The epitope recognized by 6B11 MAB is highly immunogenic since it induces the B-cell response in all patients investigated with identified anti-NS4 antibodies in blood serum. The MAB panel obtained in this study may become a useful tool for the diagnostic purposes, for the investigation of NS4B function and for the host-viral interactions at the cell level.
Key words: *Hepatitis C virus; NS4 protein; Monoclonal antibodies; Epitope mapping*

1. Introduction

Hepatitis C virus (HCV) is one of the major etiologic agents of nonA, nonB hepatitis which induces a wide range of chronic liver diseases, liver cirrhosis and hepatocellular carcinoma. HCV contains a positive sense, single stranded RNA genome of ~ 9500 nucleotides.
The genome carries a single open reading frame encoding a polyprotein of about 3010 amino acides which is proteolytically cleaved into at least ten viral structural and nonstructural (NS) proteins (Fig. 1) [1].

The nonstructural NS4 protein plays an important role in the viral life-cycle and in the development of an immune response to HCV. In fact, the NS4A protein acts as a cofactor for the NS3 serine protease cleaving the precursor polyprotein into NS3/NS4A, NS4A/NS4B and NS4B/NS5A proteins [1]. The functions of NS4B are still remain unknown; however, it may be important in viral replication and assembly [2].

The NS4 protein has been used as an antigenic target in various commercial diagnostic tests for the detection of HCV antibodies in the serum of patients with HCV infection. A recombinant C-100-3 protein containing the 5-1-1 immunodominant region in this protein have major diagnostic relevance [3,4]. There is evidence suggesting the possibility of using NS4 as a viral

replication marker [5,6]. The correlation between the activity of anti-NS4 antibodies and the responses to interferon treatment [7,8] suggests that detection of NS4 is useful in the evaluation of the effectiveness of antihepatitis C immunotherapy.

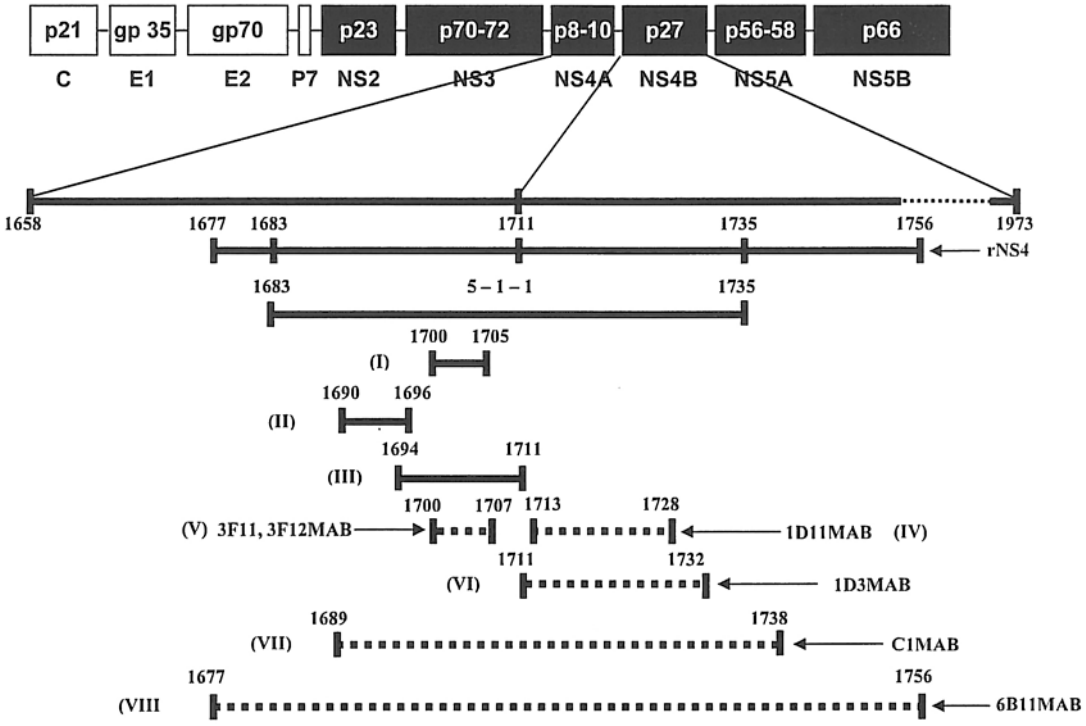

Fig. 1. Epitope mapping of 5-1-1 region of the HCV NS4 protein by monoclonal antibodies. Schematic representation of HCV organization: structural (open boxes) and nonstructural (filled boxes) proteins. rNS4 - recombinant protein used for immunization and MAB preparation. Epitopes, detected by: I-Gonzalez-Peralta et al., 1995 [7]; II and III, Sansonno et al., 1995 [28]; IV – VIII - this study. Numbers indicate amino acid position in the HCV NS4 protein.

The NS4 protein is one of the most variable of HCV proteins, with the amino acid sequence varying considerably between genotypes [9,10], which is reflected in genotype-dependent activity of anti-NS4 antibodies from serum of HCV patients [11]. Investigations with synthetic peptides derived from the NS4 protein and with panels of sera from HCV patients have revealed several immunodominant antigenic determinants on the NS4 molecule [9,10,12-15]. Monoclonal antibodies (MAB) were used for the identification of antigenic determinants of native NS4 [5,7,16,17]; however, the data on the epitope specificity of these antibodies is scarce or absent. At the same time information regarding the fine antigenic structure of the native NS4 protein is necessary to improve diagnostic methods, to develop effective anti-HCV preparations and vaccines and to better understand the pathogenetic mechanisms of the liver cell damage.

2. Materials and methods

2.1. Cloning, expression and purification of recombinant NS4 protein

Serum from a patient with high titre HCV RNA (genotype 1b) was used to amplify cDNA from the NS4 region. A fragment of cDNA corresponding to nucleotides 5363 - 5600 was synthesized using the following primers: 5'-AGCGTGGTCATTGTGGGCAGGA-3' and 5'-AAGGGCTCGCCACTTGGACTCC-3'. The gene fragment was reamplified by PCR with the same primers possessing sites for BamHI and EcoRI endonucleases and inserted into vector pGEX-4T. The NS4 protein was then expressed in *Escherichia coli* JM 109 cells.

Recombinant protein was purified by affinity chromatography [18] on gluthathione-Sepharose 4B (Pharmacia Biotech.). The final product (rNS4) was a polypeptide with the N-terminal part represented by the fusion protein gluthathione-S-transferase (GST) and the C-terminal part represented by the sequence of HCV NS4 protein from position 1677 to 1756 amino acid (aa) residues.

A panel of 33 oligopeptides overlapping NS4 sequences of region 5-1-1 from position 1683 - 1750 aa was used (Table 1). The majority of the peptides corresponded to the HCV genotype 1 (subtypes 1a or 1b); variants corresponding to other genotypes were synthesized for 1683-1705, 1689-1708, 1710-1728 and 1711-1732 aa peptides. The peptides were synthesized in accordance with reported sequences [9,10] by a previously described solid-phase method [19]. The peptides were purified by reverse-phase high performance liquid chromatography. The peptide amino acid composition was confirmed by qualitative amino acid analysis. Antigenic activity of the peptides was confirmed using sera from patients infected with HCV [20].

Table 1
Immunoenzyme analysis of monoclonal antibodies reactivity with synthetic peptides

Region of NS4 protein	Peptides, aa	Genotype	Relative activity of MAB in E1A[a]					
			3F11	3F12	1D11	1D3	6B11	C1
rNS4	1677–1756	1b	+++	+++	+++	+++	+++	+++
5-1-1	1689–1738	1b	+++	+++	+++	+++	+	+++
NS4 A	1683–1705	1b,2a,2b, 3a	–	–	–	–	–	–
	1688–1705	1b	±	±	–	–	–	–
	1689–1707	1a	+++	+++	–	–	–	–
	1689–1708	1a	+++	+++	–	–	±	–
		1b	+++	+++	–	–	±	–
		2a	++	++	–	–	±	–
		2c	++	++	–	–	–	–
		3a	++	++	–	–	–	–
	1693–1707	1b	+++	+++	–	–	–	–
	1700–1711	1a	+++	+++	–	–	+	±
	1702–1708	1a, 1b	–	–	–	–	–	–
NS4 B	1710–1728	1a	–	–	+++	–	–	–
		2b, 2c, 4, 5, 6	–	–	–	–	–	–
	1711–1732	1a	–	–	+++	–	–	–
		1b	–	–	+++	+++	–	–
		2a, 2b	–	–	–	–	–	–
		3a	–	–	++	–	–	–
	1713–1731	1b	–	–	+++	+	–	–

[a] Signal/phone ratio (P/N) at purified Ig concentration 1 ug/ml; –, < 30; ±, 3–5; +, 6–10; ++, 11–30; +++, > 30.

2.3. Production of monoclonal antibodies

BALB/c female mice were immunized intraperitoneally four times at 2-week intervals with 20 ug of purified rNS4 mixed with an equal volume of Freund's complete adjuvant. Three days before hybridization the mice were boosted by intraperitoneal injection of 20 ug rNS4. Hybridization of the mouse splenocytes with the Sp2/0 myeloma cells was performed as described elsewhere [21].

Cell clones were selected by indirect enzyme immunoassay (EIA). Three antigens (rNS4, 0.5 ug/ml; *E. coli* lysate, 10 ug/ml; GST, 1 ug/ml) were coated onto the 96-well plates (Nunc) in PBS, pH 7.4, overnight at a room temperature. Incubation with cultural fluid from hybridomas (HCF) at a 1:50 dilution in PBST (PBS with 0.1% Tween 20) was carried for 1 h at 37°C, then anti-mouse horse radish peroxidase (HRPO) conjugate specific for IgG (A4416, Sigma) in PBST with 20% fetal serum was added. OPD (Sigma) was used as a substrate. Optic density (OD) was measured at 492 nm. HCF unreactive with *E. coli* proteins and GST and giving OD>1.0 with rNS4 were considered as positive. Six hybridomas were selected and cloned by the method of limiting dilutions.

2.4. Purification of monoclonal antibodies

The properties of the obtained MAB were studied using HCF and Ig purified from ascitic fluids by affinity chromatography on a Protein A-Sepharose CL-4B column (Pharmacia) according to the manufacturer's instructions. The protein concentration was measured using Bio-Rad Protein Assay kit. Subtyping and the light chain typing were performed with Mouse-Hybridoma Subtyping kit (Boehringer Mannheim). Purified MAB were conjugated with HRPO by the periodate method [22]. The antibodies were used in the following assays.

2.5. EIA and immunoblot

Anti-rNS4 activity of MAB was determined by a solid-phase EIA. The MAB were also screened in a Murex HCV ELISA (version 3) and RecombiBest anti-HCV ELISA confirmatory assay (Vector-Best, Russia) where the kit anti-human conjugate were replaced with the anti-mouse conjugate (A4416, Sigma). Immunoblot with Chiron recombinant immunoblot assay (RIBA) HCV test system 3.0 (RIBA 3) was performed according to the manufacturer's recommendations, excluding the replacement of anti-human kit conjugate by anti-mouse conjugate.

2.6. Epitope mapping and reciprocal competitive analysis

Epitope mapping was carried out in EIA. The peptides were absorbed on a solid phase of 96-well plates (Nunc) in a concentration of 10 ug/ml in PBS for 2 h at 37°C, and nonspecific binding sites were blocked with 2% BSA. Anti-mouse conjugate was added after 2-h incubation at 37 °C (undiluted HCF or 1 ug/ml purified Ig in PBST with 20% fetal serum). The results were considered positive when OD was at least three times as high as that in negative controls-MAB to HCV core protein [23]. Antigenic specificity of MAB was compared in competitive EIA. The HRPO-conjugated MAB in dilutions giving OD 1.0 - 1.5 were incubated in wells coated rNS4 together with purified nonlabeled antibodies in a concentration of 100 ug/ml. The results were expressed as inhibition degree of the binding of the conjugated MAB with rNS4 in the presence of nonlabeled MAB. The competitive reactivity of MAB with anti-HCV antibodies in human sera was assayed. The reaction was performed as described above excluding the replacement of nonlabeled MAB by human anti-HCV sera at a dilution 1:20.

2.7. Immunohistochemical staining

In order to assess the ability of the MAB to react with native antigen, liver samples obtained by needle biopsy from 22 anti-HCV-positive (Ortho HCV 3,0 ELISA test System) patients with chronic hepatitis C (CHC) were analyzed for the presence of HCV antigens by immunohistochemical staining (IHS). Biopsies were performed for diagnostic purposes, and informed consent was obtained from each patient. All patients had histologically proven chronic hepatitis: minimal inflammation (histological activity index, HAI 1-3) was seen only in 1 biopsy, mild inflammation (HAI 4-8) was seen in 11 biopsies, and moderate inflammation (HAI 9-12) was seen in 10 biopsies (categories according to [24]). All biopsies showed some amount of compacted portal fibroses indicative of a chronic stage of hepatitis.Cryostat liver sections (5 um) were fixed with acetone/chloroform mixture (1:1) for 10 min and incubated with purified primary MAB (10 ug/ml in 1% BSA/PBS) for 1h after blocking of endogenous peroxidase with 0.6% hydrogen peroxide in methanol for 20 min. The sections were then incubated with secondary biotinylated goat anti-mouse immunoglobulins for 30 min and peroxidase-labelled streptavidin (DAKO corp., Cat. No. K0672) for 30 min. Each incubation with immune reagents was carried out at room temperature and followed by a wash in three changes of PBS. 3-Amino-

9-ethyl-carbazole (AEC) was used as an HPRO substrate. The sections were counter-stained with Mayer's hematoxylin for 5 min. On the resulting slides, the sites of immunoperoxidase activity were stained red and nuclei were blue. Both the number of stained cells and the intensity of staining were taken into consideration upon interpretation of the results.

Four control reactions were performed: (a) substitution of an equivalent amount of irrelevant MAB (MAB to p24 protein of HIV-1) for the primary antibody, (b) omission of the primary MAB, (c) use of primary anti-NS4 MAB that had been preabsorbed with rNS4 protein, (d) study of biopsies from eight anti-HCVnegative, HCV-RNA-negative patients (hepatitis B virus, one case, and seven uninfected individuals with other forms of chronic liver disease: cholestatic hepatitis, Wilson's disease, etc.)

2.8. Detection of HCV RNA

The positive and negative HCV RNA chains were detected by nested reverse transcriptase polymerase chain reaction (RT-PCR) using synthetic primers from the 5'-untranslated region of HCV genome [25,26].

3. Results

3.1. Antigenic and immunogenic properties of recombinant NS4 protein

Recombinant NS4 protein, which was used in the present study, was characterized for sensitivity and specificity of antibody detection in EIA with a panel of 18 donor sera, ten of which were HCV-positive and eight were HCV-negative according to the Murex HCV ELISA test. Recombinant NS4 recognized anti-HCV antibodies in nine out of ten sera and gave no false positive results. Negative result in one case was caused by the absence of anti-NS4 antibody detected by RIBA-3 (Table 2, specimen 10). Immune sera with high activity of specific antibodies (EIA titer 10^{-8}) were obtained after immunization of mice with rNS4. In the RIBA 3 test-system the mice antisera reacted with a band that corresponded to the peptides from the NS4 protein (data not shown).

Table 2
Competition of monoclonal antibodies with anti-HCV antibodies in the blood sera

Serum		Activity in EIA Murex a-HCV[a]	Interaction with NS4 in RIBA 3	Inhibition					
				Conjugates of MAB					
				3F11	3F12	1D11	1D3	6B11	Cl
Anti-HCV-positive	1	2.1	+	++	+++	+++	++	++	+
	2	1.2	+	++	++	+++	++	++	+++
	3	0.9	+	+	++	+++	++	++	+
	4	1.9	+	+	+	++	++	+	+
	5	1.5	+	+	+	++	+	+	+
	6	1.4	+	+	+	++	+	+	−
	7	1.3	+	−	−	+	−	++	+
	8	2.0	+	−	−	+	++	+	+
	9	1.8	+	+	+	+	−	+	−
	10	1.8	−	−	−	−	−	−	−
Anti-HCV-negative	11–18	< 0.7	−	−	−	−	−	−	−

[a] OD_{450} at dilution of serum 1:10; + + +, 100 - 75% inhibition; + +, 74 - 50% inhibition; +, 49 - 25% inhibition; -, < 25% inhibition.

3.2. Characterization of anti-NS4 MAB

We have obtained more than 400 hybridoma clones, 200 of which reacted with rNS4 but not with GST and *E. coli* proteins. Six stable hybridomas were selected and cloned. The characteristics of MAB secreted by these hybridomas are listed in Table 3. All hybridomas produced class G immunoglobulins. Upon interaction with rNS4 in EIA, 3F11 and 3F12 MAB exhibited the highest activity, while 1D3 and C1 MAB had the lowest activity. Four MAB reacted positively with the recombinant antigen in EIA (Murex HCV ELISA) and 5 MAB were positive in Vector-Best test-system and interacted with peptides from NS4 region in RIBA 3 (Fig. 2 and Table 3).

Table 3
Characteristics of monoclonal antibodies to recombinant NS4 protein of hepatitis C virus

MAB	Subtype Ig, light chain	EIA with rNS4, titre	Activity in EIA test-systems		Reaction with NS4 band in RIBA 3	Reaction with peptides	
			Murex a-HCV[a]	Vector-Best a-HCV[a]		Sequence, aa.	Genotype
3F11	IgG1, k	5×10^{-7}	> 3.0	> 3.0	+	1700–1707	1a, 1b, 2a, 2c, 3a
3F12	IgG1, k	9×10^{-7}	> 3.0	> 3.0	+	1700–1707	1a, lb, 2a, 2c, 3a
1D11	IgG2b, k	5×10^{-6}	–	2.9	+	1713–1728	1a, lb, 3a
1D3	IgG1, k	10^{-4}	2.2	0.67	+	1711–1732	1b
6B11	IgG1, k	10^{-6}	0.31	0.84	+	1677–1756	N/I
C1	IgG1, k	10^{-5}	–	–	–	1689–1738	N/I

[a] OD_{492}, OD_{492} of negative control, 0.1; –, no reactivity; N/I, not identifed.

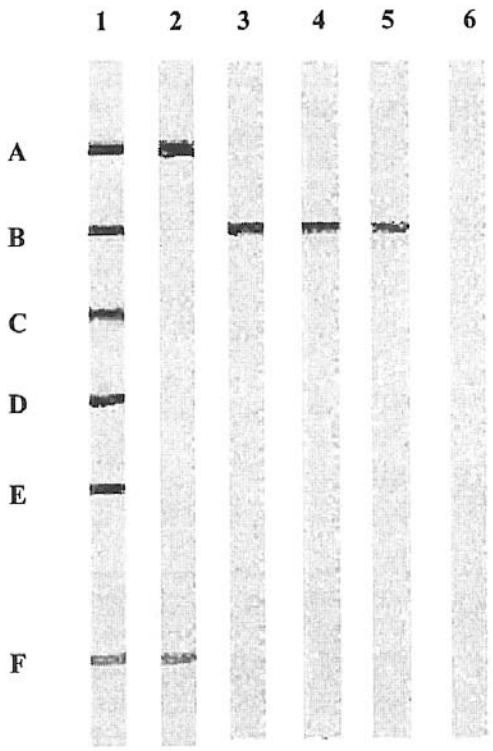

Fig. 2. Immunoblot analysis of monoclonal antibodies interaction with HCV antigens in RIBA 3 test-system. A, strip control; level II, B, 5-5-5; C, 100 (peptides, NS4); C, c33c (recombinent NS3); D, c22 (peptide, core); E, recombinant NS5; F, strip control, level I; Lane 1, kit positive control; Lane 2, kit negative control; Lane 3, MAB 3F12; Lane 4, MAB 1D11; Lane 5, MAB 6B11 and Lane 6, MAB C1.

3.3. Epitope specificity of MAB

We assessed the ability of the MAB to interact in EIA with peptides adsorbed on solid phase (Table 1). Each MAB actively bound to the 50-mer peptide from the sequence 1689 - 1738 aa (5-1-1 antigenic region); 6B11MAB showed the lowest activity. None of the four peptides from the 1683 - 1705 sequence reacted with any MAB. 3F11 and 3F12 MAB had similar epitope specificity and actively bound to a number of peptides from the 1688 - 1711 sequence, including the peptides corresponding to HCV genotypes 1, 2, and 3. 1D3 MAB actively bound only to a 22-mer peptide (1711 - 1732) containing the genotype 1b sequence. 1D11 MAB reacted with the same activity with both 1a and 1b subtype peptides from the 1711- 1732 sequence and with lower activity with peptide of 3a genotype from the same region. The MAB 1D11 also actively bound shorter 19-mer peptides (1713 - 1731 and 1710 - 1728 aa) and did not react with peptides of the HCV genotypes 2, 4, 5 and 6. We failed to determine epitope specificity of C1 and 6B11 MAB with the peptides. C1 MAB actively reacted only with 50-mer peptide from the sequence 1689-1738 aa. 6B11 MAB also showed the highest affinity for this peptide, however, the intensity of the interaction of the MAB with this peptide was significantly lower than with rNS4. Weak signals were also obtained with some peptides from the 1689 - 1711 region. Five peptides (1716 - 1731, 1720 - 1739, 1724 - 1743, 1728 - 1747 and 1732 - 1750) did not react with any MAB and are not included in Table 1.

3.4. Competitive analysis of MAB

Five of the six MAB were able to inhibit themselves from binding to rNS4 coated solid-phase (Fig. 3). Full reciprocal blocking was observed with 3F11 and 3F12 MAB.

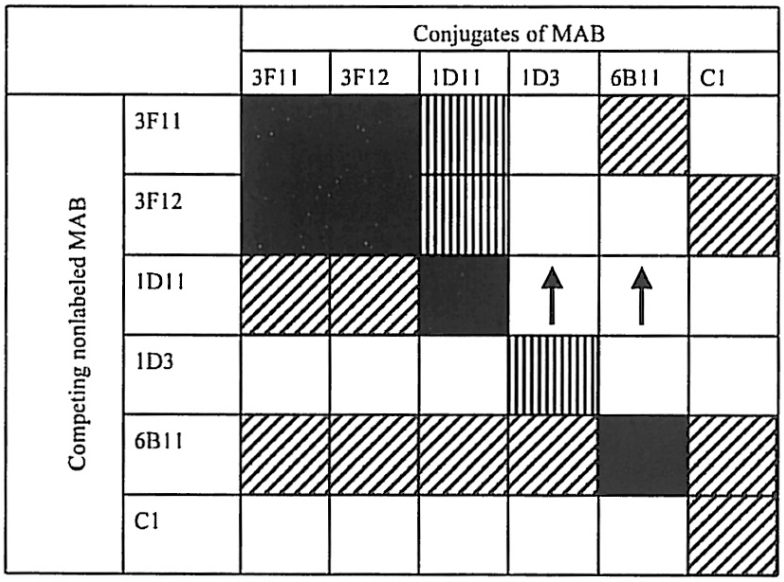

Fig. 3. Competitive immunoenzyme analysis of MAB interaction with rNS4. Binding of MAB conjugates with rNS4 inhibited of: ■ , 100 - 75%; □ 74- 50%; □ 49 - 25%; □ < 25%;↑ increase of binding.

3F11 and 3F12 MAB showed limited crossblocking of the 1D11 MAB and competed differently with conjugates of 6B11 and C1 MAB. 6B11 MAB inhibited the binding of all conjugated MAB to rNS4 by 25 - 49%; however, with the majority of the antibodies the inhibition was unilateral. When 1D11 MAB was used as competing agent, the binding of conjugated 1D3 and 6B11 MAB to rNS4 increased by 50 - 70%. 1D3 and C1 MAB weakly inhibited the antigen-antibody binding.

The ability of the MAB to compete with anti-NS4 antibodies from sera of patients with chronic hepatitis C were compared by competitive EIA. All studied MAB could compete with patients antibodies for binding to rNS4 (Table 2). 1D11 and 6B11 MAB inhibited anti-NS4 binding to NS4 in all sera containing antibodies to this protein.

3.5. Detection of HCV antigens in the liver of patients with chronic hepatitis C

The ability of the MAB to react with native NS4 was assessed on cryostat liver sections from 22 anti-HCVpositive patients with chronic hepatitis C (CHC). All the MAB (C1 MAB was not studied) interacted with NS4 at various intensities. It was shown that 3F11 and 1D3 MAB stained the same samples as 3F12 and 1D11 MAB, respectively, however in the most cases the staining was less intensive. Serial slides from group of 22 patients were analyzed with the use of each of 3 MAB (3F12, 1D11 and 6B11) simultaneously. The positive rates of the immunostaining for NS4 antigen by MAB 3F12, 1D11 and 6B11 were 50, 59 and 64%, respectively (Table 4). The number of antigen-positive cells varied considerably (3 - 80% stained cells) in the liver sections, mean values being 30 - 44%. Staining with 6B11 MAB was mainly more intense than that with 1D11 and 3F12 MAB. In the majority of patients, positive staining with different antibodies had similar location in serial sections, differing in the number of stained cells and reaction intensity.

All of the studied MAB stained viral antigens only in the hepatocyte cytoplasm, liver cell nuclei were never stained (Fig. 4A - C). Immunoreactive cells showed a diffuse cytoplasm signal (Fig. 4C), otherwise a focal positivity distributed in distinct areas of the cytoplasm was found (Fig. 4B). Generally, MAB 3F12 and 1D11 produce fine-granular staining of NS4 antigen (Fig. 4B and C), while mesh-like granular staining was typical of MAB 6B11 (Fig. 4A).

Positive staining was not observed after prior absorption of primary MAB (3F12, 1D11 and 6B11) with purified rNS4. Anti-HIV-1 MAB (negative control) produced no background staining on the cryostat sections. Each of the NS4-specific MAB was tested on liver sections from eight HCV-negative patients. None of the MAB produced typical staining (Fig. 4D).

Study of positive (genomic) and negative (replicative) HCV RNA chains in 22 anti-HCV-positive patients has detected positive RNA chains in hepatocytes of 20 patients and negative RNA chains only in 12 patients.

Table 4
Immunohistochemical detection of NS4 antigen in liver sections of patients with chronic hepatitis C by MAB

Parameters of immunohistochemical staining	Reaction with MAB		
	3F12/3F11	1D11/1D3	6B11
Number of patients with positive results	11/22 (50%)	13/22 (59%)	14/22 (64%)
Number of stained cells (%)			
Mean	30	36	44
Range	3–70	5–70	15–80
Comparative intensity of stained samples (%)			
High	18	23	21
Medium	18	31	50
Week	64	46	29

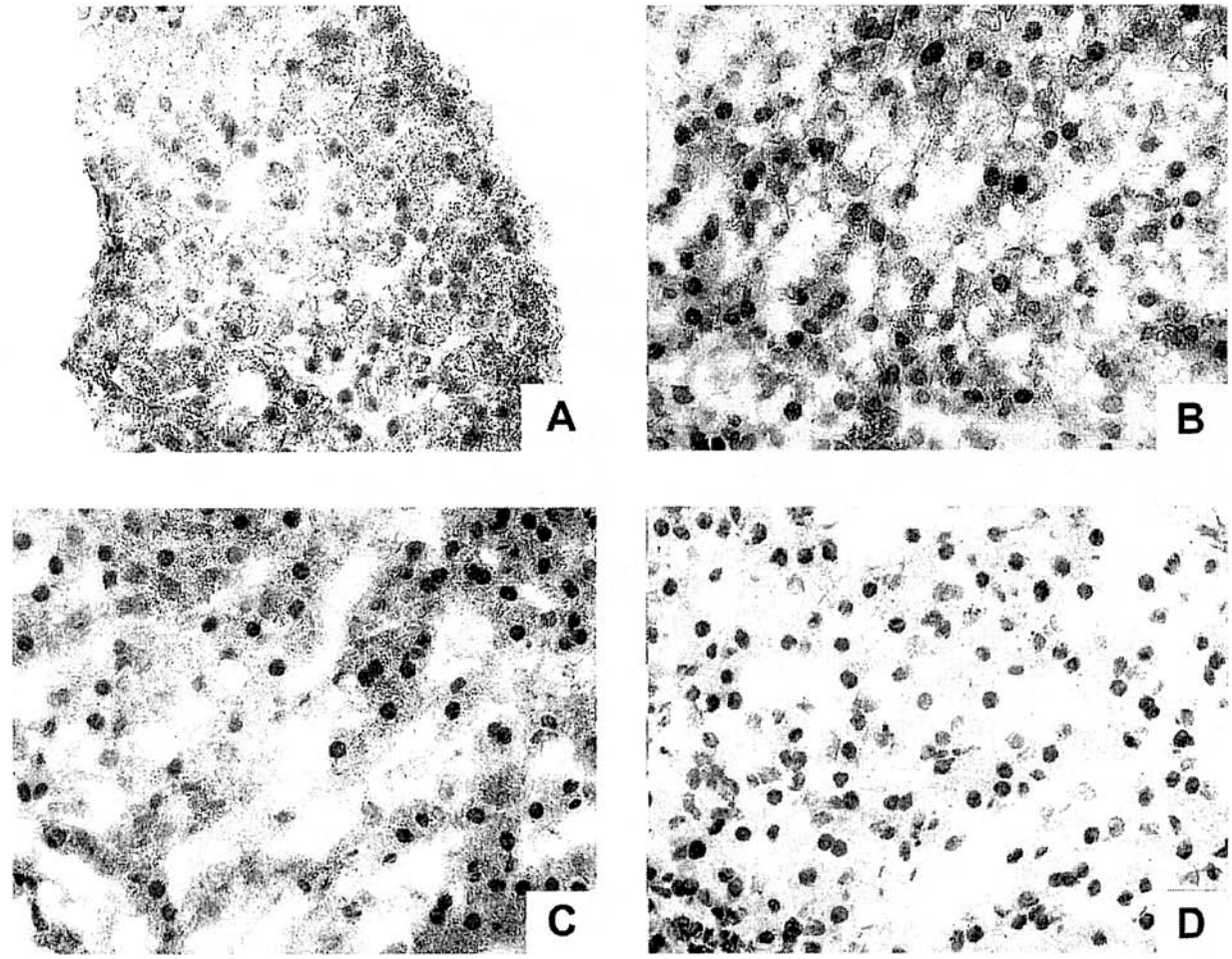

Fig. 4. Immunohistochemical detection of HCV NS4 antigen in cryostat liver sections. (A) Staining with MAB 6B11. Note intensive granular mesh-like immunoreactivity in the cytoplasm of most hepatocytes (patient B. with moderate CHC). Magnification, x 400. (B) Staining with MAB 3F12.Immunoreactive NS4A-containing cells showed a diffuse and focal cytoplasmic signal in the form of fine granules (patient M. with moderate CHC). Magnification, x 400. (C) Staining with MAB 1D11. NS4B antigen is diffusely distributed in the cytoplasm in the form of fine granules (patient O.with mild CHC). Magnification, x 400. (D) Staining with MAB 6B11 of liver section from anti-HCV-negative and HCV-RNA-negative patient Ch.with chronic hepatitis B (negative control). No immunoreactivity is observed. Magnification, x 400

4. Discussion

The recombinant NS4 that was expressed in *E. coli* exhibited high antigenic activity: it reacted with all of sera from HCV-positive patients contained the anti-NS4 antibodies in EIA and immunoblot. The immunogenic properties of rNS4 were confirmed by high activity of polyclonal sera obtained after immunization of mice and by large number of NS4-specific hybridoma clones (200 out of 400).

A comparative analysis of the interaction of the MAB with synthetic peptides allowed mapping of the antigenic epitopes of the NS4 protein. The minimal linear epitope recognized by 3F11 and 3F12 MAB is located in the 1700 - 1707 region of NS4A protein (Tables 1 and 3). It is likely that 3F11 and 3F12 MAB recognize the same linear epitope, as evidenced by results of EIA, immunoblot and peptide mapping. Minor differences were observed between these clones only in competitive assays (Fig. 3 and Table 2) and in the efficiency of natural antigen recognition in the liver sections. These differences are probably associated with idiotypic differences between immunoglobulin molecules. The key amino acid residues were found to be located in positions 1701 and 1702 [14] and 1703 [12]. It should be noted that 3F11 ad 3F12 MAB were practically unreactive with peptides from the sequences 1688 – 1705 and 1702 -

1708 in solid-phase EIA, but these peptides in solution were more effective in inhibiting MAB binding to the rNS4 (data not shown). Thus, these sequences require additional flanking residues for optimal recognition of the epitope on solid phase. Monoclonal antibodies reacting with 1700 - 1705 peptide and recognising only genotype 1 (1a and 1b) sequences have been described [7]. Our findings show that together with a genotype-specific epitope at position 1700 - 1705 aa, the C-terminal part of the NS4A protein contains an antigenic determinant common for genotypes 1 - 3. Other researchers came to a similar conclusion after demonstrating that 1691 - 1708 peptides react with sera from patients infected by various genotypes of HCV [9,15].

Two MAB (1D11 and 1D3) proved to be specific to another immunodominant region of NS4; namely, the N-terminal part of the NS4B protein that includes 1711- 1728 amino acid residues. The epitopes recognized by these MAB were different. This was confirmed by the findings of EIA, peptide mapping and competitive assay. 1D3 MAB reacted only with 1711 - 1732 peptide of 1b subtype, while 1D11 MAB reacted with similar peptides of 1b, 1a and 3a subtypes as well as with shorter peptides (1713 - 1732 and 1710 - 1728). Thus, the minimal antigenic determinant recognized by 1D11 MAB was identified within the sequence 1713 - 1728. 1D11 and 3F11/3F12 MAB partially blocked each other in the competitive assay. This blocking is probably of steric nature and results from close location of the recognized epitopes. Thus, epitopic mapping of the Nterminal part of NS4B with our MAB revealed at least two antigenic determinants, one of which (recognized by 1D3 MAB) proved to be not only genotype- but also 1b subtype-specific and other (recognized by 1D11 MAB) - genotype-independent.

We failed to determine epitope specificity of 6B11 and C1 MAB with the peptides. The anti-NS4 reactivity of 6B11 MAB decreased considerably on denaturation of rNS4 by sodium dodecyl sulfate and 2-mercaptoethanol (data not shown). These findings suggest that 6B11 MAB recognized a conformational or discontinuous epitope. It is likely that C1 MAB also recognized a conformational epitope in the 5-1-1 region; however, experimental data indicate that this determinant differs from that recognized by 6B11 MAB. It should be noted that conformation-dependent antigenic determinant of NS4 protein have not been described. The existence of these determinants was indirectly confirmed by the finding that a 50-mer peptide reacts with a considerably greater number of sera from HCV-infected patients in comparison with a mixture of two short peptides of the same sequence [20].

Some MAB and polyclonal sera to recombinant proteins and synthetic HCV peptides do not react with native viral antigens [5,7,16]. In the present study at least five MAB (C1 MAB was not tested) recognized NS4 in liver cells from patients with chronic hepatitis C. The specificity of MAB was confirmed by a series of control experiments. The active in EIA MAB (3F12, 1D11 and 6B11) bound to native NS4 with different efficiency, judging from the number of stained cells and intensity of staining. 6B11 MAB stained hepatocytes more intensively and more frequently than other two MAB. This suggests that the conformational determinant recognized by these MAB is most exposed in the native NS4 protein. The detection rate of NS4 antigen (64%) was similar to that obtained with other anti-NS4 MAB [7,17] and was superior to that [27]. Negative results of IHS in 36% biopsies can be due to small size of some samples (two to three portal tracts) or low replication of HCV in the liver The latter suggestion is supported by the facts that negative RNA chains were not detected in hepatocytes of eight (40%) out of 20 CHC patients, while positive RNA chains were detected.

Positive staining for the NS4 antigen was confined to the hepatocyte cytoplasm, which is consistent with the majority of available data [7,16,17,28,29]. However, some scientists revealed NS4 antigen mainly in the liver cell nuclei [27,30]. In both works commercial MAB to the NS4 (C-100-3) protein TORDJI 22 and TORDJI 32 were used, the epitopic specificity of which was not shown. In another work using the same MAB the cytoplasmic staining of liver cells was observed (17). Although some researchers have obtained false positive results with these antibodies and doubt their specificity [31,32], nuclear phase of replication or persistence of HCV cannot be ruled out. On the other hand, the discrepancies between the results may depend on the

method used. In the works [27,30] paraffin sections of liver tissue were used, while we used cryostat liver sections.

The data on the competition of MAB with antigens from sera of HCV-positive patients show that all prepared MAB and antibodies to native NS4 protein react with the same epitopes. This points to the exposure and high immunogenicity of epitopes recognized by the MAB. The epitopes recognized by 6B11 and 1D11 MAB are the most accessible. Thus the results on the competition agree with the data on NS4 identification in hepatocytes with the use of these MAB. Our results show that the set of epitopes reacting with human antibodies differs considerably from patient to patient. This finding is of practical significance, providing for a means of improving the technique of hepatitis diagnostics by including into a test system the entire range of antigenic determinants of NS4 inducing immune response. The differences in the interactions of 3F12 and 6B11 MAB with recombinant and native NS4 protein (Tables 2 - 4) indicate the presence of the differences in the antigenic structure of these proteins. Interestingly, in native protein the conformational epitope recognized by 6B11 MAB is the most exposed and immunologically active.

Previously, three antigenic epitopes were identified in the 5-1-1 region with the use of Mab (Fig. 1). Anti-NS4 MAB, which were characterized in the present study, recognize at least five new antigenic determinants: one linear epitope from the 1700 - 1707 sequence of NS4A, two linear epitopes from the 1711 - 1732 sequence of NS4B and two conformation-dependent epitopes in 5-1-1 region. Both genotype (subtype)-specific and genotype-independent epitopes have been mapped in N-terminal part of NS4B. This indicates a more complex antigenic structure and topology of antigenic determinants in the variable region 5-1-1 than that described previously. The MAB with high efficiency recognized native NS4 in infected hepatocytes of patients with chronic hepatitis C. The MAB panel obtained in this study may become a useful tool for both diagnostic purposes and for the investigations NS4B functions and host-viral interactions at the cell level.

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