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Antigenic Structure of the Complete Nonstructural (NS) 2 and 5 Proteins of Hepatitis C Virus (HCV): Anti-HCV NS2 and NS5 Antibody Reactivities in Relation to HCV Serotype, Presence of HCV RNA, and Acute HCV Infection

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Antigenic regions within the nonstructural (NS) 2 and 5 proteins of hepatitis C virus (HCV) were identified and characterized by the use of 127 overlapping synthetic peptides and a serum panel consisting of 167 human serum samples from persons with antibodies to HCV. Initially, 20 anti-HCV-positive serum samples were used to screen the peptides covering the complete NS2 and NS5 proteins. Among the 27 overlapping peptides spanning the NS2 protein of HCV, only the peptide covering residues 960 to 975 was recognized by human sera. Within the 100 peptides covering the NS5 protein, major linear antigenic regions were located at residues 2284 to 2329 within the putative NS5a and at residues 2584 to 2599 and 2944 to 2959 within the putative NS5b. Additional minor linear antigenic regions were also identified within the NS5. The sequence of the antigenic region of the NS2 protein is, unlike most parts of the NS2 protein, highly conserved among the described types of HCV, whereas the sequence of the major antigenic region of NS5 shows variability among HCV types. The recognition of a peptide corresponding to a part of the major region of NS5 was found to be dependent on HCV type. In 129 anti-HCV-positive serum samples, the prevalence of antibodies to the NS2 protein was found to be 23% among HCV RNA-positive sera and 10% among HCV RNA-negative sera. In the same samples, reactivity to the major linear antigenic regions of HCV NS5 was found in 68% of the HCV RNA-positive sera and 67% of the HCV RNA-negative sera. Of 18 serum samples from five patients with acute HCV infections, and who seroconverted with respect to anti-HCV, 4 were found to be reactive to one or more of the 100 NS5 peptides and in three serum samples the NS5 reactivities were found to shorten the time for serodiagnosis of HCV compared with second-generation assays. We also found that antibodies reactive to the NS2 peptide were cross-reactive with a region from residues 2584 to 2599 of NS5, which has 67% homology with a six-residue sequence of NS2. In conclusion, in this study we have identified and evaluated the potential use of synthetic peptides corresponding to linear antigenic regions of the NS2 and NS5 proteins.

The 10,000-nucleotide RNA genome of the hepatitis C virus (HCV) has been found to code for at least six different proteins (4). On the basis of homology with the flavi- and pestiviruses the HCV genome codes for one large polyprotein which is posttranslationally cleaved into the respective structural (core, envelope 1, and envelope 2) and nonstructural (NS) proteins (NS2 to NS5). The NS2 protein is a mainly hydrophobic protein for which the function and intracellular localization during translation have not been determined (16). Antibodies to this protein have so far not been described. The NS3 protein could possibly serve as a serine-protease or -helicase, which assist in the cleavage of the precursor polyprotein (18, 20). The function of the NS4 protein is still unclear, but it was the first protein encoded by HCV to be used in the serodiagnosis of HCV (8). The NS5 protein is probably the RNA-dependent RNA polymerase of HCV (18).

Antigenic regions recognized by human antibodies have been localized within the core (7, 11, 13, 14), envelope 1 (5, 15), envelope 2 (21), and the NS4 protein (15, 17). No complete antigenic map of the antigenic regions within NS2, NS3, or NS5 has yet been reported. However, the antigenicities of the majority of the HCV-coded proteins, except for the NS2 protein, have been determined by using recombinant proteins (3).

Our aim was to produce an antigenic map of the complete NS2 and NS5 translation products and to evaluate the potential usefulness of the identified linear antigenic regions with a large panel of well-characterized human sera.

MATERIALS AND METHODS

Patient sera. Twenty anti-HCV-positive serum samples were derived from a serum panel previously described (13–15). All sera had previously been screened by peptides covering the complete HCV core (14), envelope 1, and parts of envelope 2, NS1, and NS4 (15). A further 132 serum samples were derived from 60 persons whose infection with HCV had been detected at blood donor screening, 14 persons with posttransfusion hepatitis, 10 intravenous-drug users, 2 hemodialysis patients, and 47 persons with an unknown epidemiological background. All of these were obtained from the Department of Virology at the Central Microbiological Laboratory of the Stockholm County Council. From the same source, an additional 18 serum samples from four intravenous-drug users and one patient with post-transfusion hepatitis, who all showed seroconversion with respect to anti-HCV, were obtained. All sera were assayed for anti-HCV by a commercial enzyme immunoassay (EIA) (second-generation EIA; Abbott, Chicago, Ill.) and a radioimmunoblot assay (RIBA) (Ortho Diagnostics, Raritan, N.J.).

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HCV RNA detection by PCR. All samples obtained from the Department of Virology at the Central Microbiological Laboratory of the Stockholm County Council were tested for the presence of HCV RNA by PCR, by using primers from the 5′ nontranslated region as previously described (22).

Synthetic peptides. A total of 127 peptide analogs covering the complete HCV NS2 (type II/1b) (18) and NS5 (type I/1a) (4) proteins were produced by using a previously described protocol (12, 14). In brief, the peptides were synthesized on 50 mg of resin ([FMOC-PAL-PEG-PS], Millipore Corporation, Bedford, Mass.) held in polypropylene bags. All amino acids were prepared in stock solutions and dispensed to the appropriate peptide bags at each coupling step. Throughout the synthesis *N,N*-dimethylformamide was used as the sole solvent for the coupling and washing steps. The final peptides were then cleaved and deprotected using trifluoracetic acid containing the appropriate scavengers (12). Peptides were lyophilized and dissolved in distilled water at a concentration of 1 mg/ml, and then purity was estimated by reverse-phase high-pressure liquid chromatography.

A peptide corresponding to the major antigenic region of the HCV core, residues 1 to 28 (14), was produced by using a continuous-flow synthesizer (Milligen 9050; Millipore Corporation) and was purified by high-pressure liquid chromatography to a purity of 99.9%.

A panel of 12 antigenic peptides representing each of the five major types of HCV, 1a, 1b, 2a, 2b, and 3 (1) (also termed types I to V [9]), was also produced. Three peptides covered residues 68 to 81 of HCV core (types I/II/1, III/IV/2, and V/3), five peptides covered residues 1691 to 1710 of NS4 (types I/1a, II/1b, III/2a, IV/2b, and V/3), and four peptides covered residues 2303 to 2319 of NS5 (types I/1a, II/1b, III/2a, and IV/2b), according to published sequences (1, 2, 4, 6, 9, 10, 18, 19).

EIAs. Peptide EIAs were performed as previously described (14). In brief, microtiter plates (Maxisorp 96F Certificate; Nunc, Roskilde, Denmark) were coated with peptides at 1 μg per well in 0.05 M sodium carbonate buffer, pH 9.6, and left overnight at room temperature. The plates were washed four times with 0.15 M NaCl containing 0.05% Tween 20. Sera were diluted 1:100 in 0.05 M phosphate-buffered saline containing 0.05% Tween 20, 2% goat serum, 1% bovine serum albumin, 2% dry milk powder, and 0.02% NaN_3. All sera were added in 100-μl portions to the microplates and were incubated for 1 h at 37°C. Bound serum immunoglobulin G was indicated by the addition of alkaline phosphatase-labelled goat anti-human immunoglobulin G (A-3150; Sigma Chemicals, St. Louis, Mo.) for 1 h at 37°C, diluted 1:1,500. The enzyme reaction was developed during 30 min by the addition of 100 μl of *p*-nitrophenyl phosphate (0.9 μg/μl; Sigma) in 1 M diethanolamine-HCl buffer, pH 9.8, containing 0.5 mM $MgCl_2$ and 0.02% NaN_3. The optical densities of the reaction products were measured at 405 nm. To obtain a high specificity in the screening assays, all reactions in the EIAs with the peptides exceeding the mean of eight negative serum samples by 7 standard deviations were regarded as positive.

All reactions in the serotyping EIAs exceeding the mean optical density at 405 nm of six negative serum samples by more than 3 standard deviations were regarded as positive.

Inhibition assays. All inhibition assays were performed as follows: prior to the addition of 50 μl of serum diluted 1:50, 50-μl portions of dilution buffer containing 10 μg of peptide were added to the wells. The assays were then continued as previously described.

In the serotyping assays, sera with reactivities to several type-specific peptides were retested with each peptide. Prior to the addition of sera, 10 μg of each type-specific peptide was added to individual wells. The reactions that could be completely inhibited by one type-specific peptide, but not to the same extent by the peptides corresponding to the other types, were considered specific for the peptide giving the highest inhibition. Theoretically, if an inhibiting peptide cannot completely eliminate the observed reactivity, this indicates that only cross-reactive antibodies have been inhibited and that the remaining reactivity is due to type-specific antibodies.

RESULTS

Antigenic regions within the NS2 and NS5 proteins. The 127 overlapping peptides covering NS2 and NS5 were screened with 20 anti-HCV-positive human serum samples. Among the 27 overlapping peptides spanning the NS2 protein of HCV, only the peptide covering residues 960 to 975 was recognized by human sera (Fig. 1). The sequence of this region of the NS2 protein is almost completely conserved between the described types of HCV (1, 9, 10). Within the 100 peptides covering the NS5 protein, three major antigenic regions were located at residues 2284 to 2329, 2584 to 2599, and 2944 to 2959, respectively (Fig. 1). Within the NS5 protein, additional peptides covering minor antigenic regions were also identified. The sequence of the major linear antigenic region of NS5 shows variability among HCV types. The type-specific differences between HCV types 1 and 2 are an Arg at position 2303 instead of an Asp, a Thr at position 2310 instead of a Val, and an Ala at position 2316 instead of a Leu (1, 9, 10).

Anti-HCV NS2 and NS5 reactivities in relation to HCV type. Of the 20 anti-HCV-positive serum samples used for mapping the NS2 and NS5 proteins, 17 were found to contain type-specific antibodies to one or more of the selected antigenic regions. Of these, 10 were of serotype 1, three were of serotype 2, one was of serotype 3, and three exhibited both serotype 1 and 3 reactivities. The reactivity to a part of the main antigenic region of NS5 was found to be dependent on the HCV type, since 8 of the 20 serum samples were reactive only to NS5 type I/1a and/or II/1b peptides and not to any of the type III/2a- or IV/2b-specific peptides. All of these eight serum samples were of serotype 1. In conclusion, the antigenic region at residues 2303 to 2319 of HCV NS5 is a candidate site for studying HCV serotypes.

Anti-NS2 and -NS5 reactivities in relation to the presence of HCV RNA. In 129 human serum samples, previously found to be reactive to HCV by ELISA (Abbott) and tested for the presence of HCV RNA, by PCR, the prevalence of antibodies to the NS2 protein was found to be 23% among those also containing HCV RNA and 10% among HCV RNA-negative sera (Table 1). In the same samples, reactivities to the antigenic regions of HCV NS5 were found to be 68% among those containing HCV RNA and 67% among those lacking HCV RNA (Table 1).

Detection of antibodies cross-reactive between the NS2 and NS5 proteins. Since the reactivity to NS2 residues 960 to 975 paralleled the reactivity to NS5 residues 2584 to 2599, we evaluated the possible presence of cross-reactive antibodies. The NS2 peptide contains the sequence DLAVAV and the NS5 peptide contains the sequence DLGVRV, indicating a homology of 67%. We performed cross-inhibition experiments using both peptides. In eight serum samples reactive to both the NS2 peptide and the NS5 peptide, the reactivity to each of the two peptides could be significantly inhibited by both the NS2 and the NS5 peptides (data not shown). These results clearly indicate that the antibodies to these two regions are cross-reactive at the peptide level.

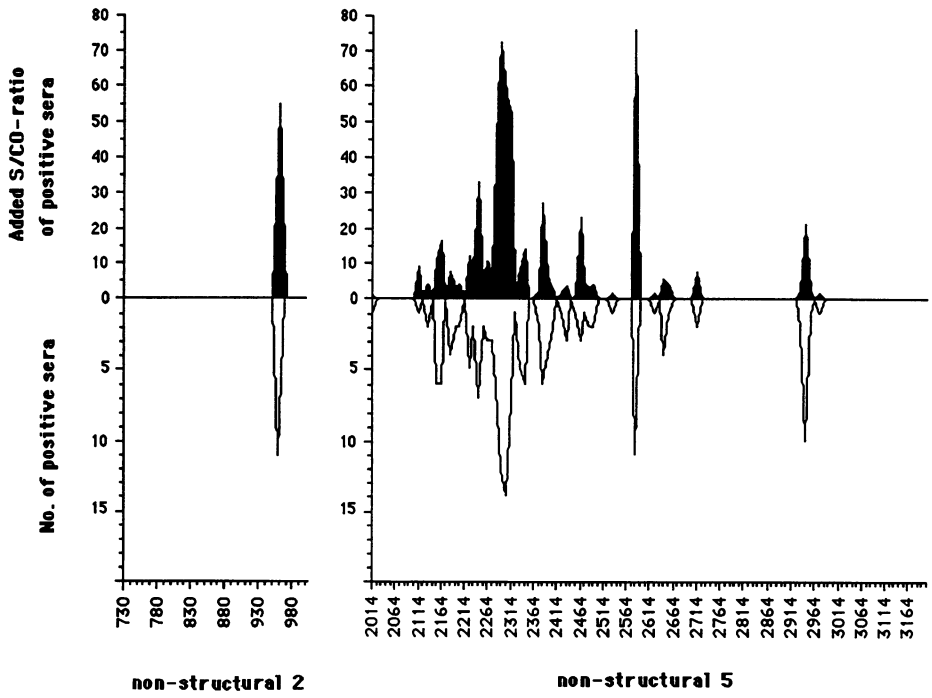

FIG. 1. Mapping of antigenic regions within the NS2 and NS5 proteins, using 16-mer synthetic peptides with a 6-amino-acid overlap, spanning the complete proteins, and 20 human anti-HCV positive serum samples. The black areas indicate the added signal-to-cutoff ratio (S/CO) of positive reactions, and the white areas indicate the number of positive reactions with each peptide.

Antibodies to NS5 in patients with acute HCV infection. Eighteen serum samples from the five patients (designated A, E, G, N, and S) with acute HCV infections, sampled during a 1-year period, were tested with the 100 overlapping peptides covering the HCV NS5 protein. In three of these patients, antibodies to one or more of the NS5 regions could be detected before seroconversion could be detected by the second-generation EIA (Abbott) and the RIBA (Ortho) (Table 2). Patient G was negative for all NS5 peptides in all samples. All positive reactivities could be verified by inhibition with the respective peptide, suggesting specific reactions.

DISCUSSION

Using overlapping synthetic peptides and a large panel of human sera, we were able to identify the major linear antigenic regions within the HCV NS2 and NS5 proteins. The mainly hydrophobic and putative membrane protein of HCV, the NS2 protein, was found to contain one major linear antigenic region. This is one of the few hydrophilic and also highly conserved regions within the NS2 protein (18). Antibodies to this region were found to be reactive irrespective of their HCV serotype. Little is known about the NS2 protein, partly because

TABLE 1. Seroreactivity to the antigenic regions of HCV NS2 and NS5 in relation to outcome in RIBA (Abbott) and presence of HCV RNA, in 129 anti-HCV positive serum samples

Outcome in RIBA	No. of serum samples	HCV RNA status (*n*)	No. of serum samples reactive to indicated region of HCV[a]								
			HCV core (1–28)	NS2 (960–975)	NS5 (2284–2299)	NS5 (2294–2309)	NS5 (2304–2319)	NS5 (2314–2329)	NS5 (2584–2599)	NS5 (2944–2959)	All NS5 peptides
Positive	84	+ (77)	69	16	17	30	20	14	26	24	53
		− (7)	7	0	0	5	1	2	2	2	6
Indeterminate	30	+ (7)	5	3	2	1	1	2	3	3	4
		− (23)	8	3	3	6	2	1	8	7	14
Negative	15	+ (0)	0	0	0	0	0	0	0	0	0
		− (15)	1	1	1	0	1	1	1	3	4
Total	129	129	90	23	23	42	25	20	40	39	81

[a] Numbers in parentheses are amino acid positions.

TABLE 2. Results from antibody and HCV RNA testing of four patients who seroconverted with respect to anti-HCV between the first and second serum samplings

Patient	Wks from first sample	Result of:		Reactivity to indicated region of the HCV NS5[a]						
		Anti-HCV EIA[b]	HCV RNA PCR[c]	2154–2189	2274–2329[d]	2494–2509	2584–2599[d]	2644–2659	2784–2799	2944–2959[d]
A	0	−	+	+	−	−	−	+	−	−
	11	+	+	+	−	−	−	+	−	−
	15	+	+	+	−	−	+	+	−	+
	49	+	+	+	−	−	+	+	−	+
E	0	−	+	−	−	−	−	−	−	−
	4	+	+	−	−	−	−	−	−	−
	24	+	+	−	+	−	−	−	−	−
N	0	−	+	−	−	+	−	−	+	−
	8	+	+	−	−	+	−	−	+	−
	34	+	+	−	−	+	−	−	+	−
S	0	−	+	−	+	−	+	−	−	+
	1	+	−	−	+	−	+	−	−	+
	6	+	−	−	+	−	+	−	−	+
	11	+	+	−	+	−	+	−	−	+
	33	+	−	−	+	−	+	−	−	+

[a] Results are only for peptides for which reactions were obtained. Ranges are amino acid positions.
[b] Determined by second-generation EIA (Abbott).
[c] Determined by PCR.
[d] Major linear antigenic region.

of the difficulty of producing this protein by using various expression systems. By using affinity-purified antibodies to this region it will be possible to detect the NS2 protein in infected and transfected cells and to study the intracellular localization by eukaryotic expression systems. However, since we could show that the antibodies to this region also cross-react with a region of NS5, care should be taken when human sera are used for identifying the NS2 and NS5 translation products (16, 20).

The NS5 protein, considered to form the putative RNA-dependent RNA polymerase of HCV, was found to contain several linear antigenic regions. The major linear antigenic regions spanning residues 2284 to 2329, 2584 to 2599, and 2944 to 2959 were all located at mainly hydrophilic regions within the NS5 protein. Affinity-purified antibodies to the region at residues 2284 to 2329 could thus be used to detect the NS5a cleavage product, and antibodies to the other two regions, especially the one at residues 2944 to 2959, could be used to identify the NS5b protein (20).

Antibodies to the linear regions of the NS2 and the NS5 proteins were found in both HCV RNA-positive and RNA-negative patient materials and were thus not found to signal either an infectious or a noninfectious stage of disease. It can, however, be suggested that high levels of antibodies to the RNA-dependent RNA polymerase are related to the replicative phase of the infection, but further investigations are needed to clarify this. According to our results it is possible that NS5 peptides might add to the sensitivity of core-based anti-HCV assays. Thus, the use of NS5 peptides may be of diagnostic value.

Of five patients who were found to be positive for HCV RNA by PCR but negative for anti-HCV by commercial second-generation assays in the first sample drawn, three were reactive to one or more of the overlapping NS5 peptides. This suggests that selected NS5 peptides could also shorten the seronegative phase in acute HCV infections.

In conclusion, in this report we describe several linear antigenic regions within the HCV NS2 and NS5 proteins. The identification of these regions has implications for the prediction of the structure of these proteins, since these regions are likely to be surface exposed in the structure of the translated proteins. These regions might also serve as peptides to be included in future anti-HCV assays, and antipeptide antibodies might be used for studying the presence and intracellular processing of HCV-coded proteins in hepatocytes and eukaryotic expression systems.

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