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Linear B-Cell Epitopes of the NS3–NS4–NS5 Proteins of the Hepatitis C Virus as Modeled with Synthetic Peptides

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A set of 150 synthetic peptides spanning the proteins NS3–NS4–NS5 of the hepatitis C virus (HCV) was synthesized and tested with a panel of 20 sera obtained from HCV-infected patients. Of 62 peptides prepared from the NS3 region, none exhibited strong antigenic reactivity. Rather, five peptides from this region demonstrated specific reactivity with only 5–10% of anti-HCV-positive sera. Nonetheless, it is well known that the NS3 region contains strong antigenic epitopes. These epitopes appear to be modeled in a functionally active manner with recombinant proteins and cannot be mimicked properly with short synthetic peptides. This finding suggests that the major NS3 antigenic epitopes are conformationally dependent. Seven of 20 peptides prepared from the NS4 region were immunoreactive. Five peptides from this region demonstrated very strong HCV-specific antigenic reactivity. Four of the five peptides belong to the recognized immunoreactive 5-1-1 region located inside the C100-3 antigen. One peptide demonstrating immunoreactivity with approximately 90% of anti-HCV-positive sera was found outside the C100-3 region at the C-terminal part of the NS4 protein. Of 68 peptides synthesized from the NS5 protein, 30 were immunoreactive. Six of the 30 demonstrated immunoreactivity with 35–50% of anti-HCV-positive sera. Thus, the NS4 and NS5 regions of the HCV polyprotein contain a large number of specific, broadly reactive, linear antigenic epitopes. The highly antigenic reactivity of the NS5 region suggests that this protein may have significant diagnostic potential.

The hepatitis C virus (HCV) is the major causative agent of parenterally transmitted non-A, non-B hepatitis worldwide (*1*). Recent progress in the identification, cloning, and sequencing of the HCV genome (*2*) allowed for the elucidation of the genomic organization of this virus. The HCV genome is a positive RNA molecule that shares homology with flavivirus and pestivirus (*3*). The complete primary structure of the genome from several HCV isolates is known (*4–13*). Preparation of HCV-specific cDNA has accelerated the development of diagnostic test systems for the identification of serologic markers of this infection. Among the most important diagnostic reagents for the detection of anti-HCV-specific activity in sera were the C33 and C100-3 recombinant polypeptides derived from the nonstructural proteins of NS3 and NS4, respectively (*14, 15*). The structural proteins, especially the nucleocapsid protein C, also contain broadly reactive antigenic epitopes (*16–20*) and have been included in various test systems (*15, 16, 18, 21–24*). Fragments of the NS5 protein expressed in *Escherichia coli* are immunoreactive with sera obtained from HCV-infected individuals (*23, 25*), a finding that suggests that this protein may also elicit diagnostically relevant antibodies. Therefore, almost all HCV-specific proteins have been reported to contain antigenic epitopes. The antigenic composition of the HCV structural proteins has been thoroughly characterized (*18, 20, 26–30*), whereas a complete analysis of the nonstructural proteins has not yet been published. Only few epitopes have been found in these proteins by using synthetic peptides (*28, 31–33*). To analyze the antigenic structure of the HCV nonstructural proteins, we prepared a set of 150 synthetic peptides spanning more than 90% of the NS3–NS4–NS5 region of the HCV polyprotein (Fig. 1). All synthetic peptides were prepared using the sequence of the HCV genome published by Kato *et al.* (*4*). Almost all the peptides were 20 amino acids (aa) long with only few exceptions when 19-mer peptides were synthesized. The NS3 protein was spanned with 62 peptides, the NS4 protein with 20 peptides, and the NS5 protein with 68 peptides. When designing peptides for chemical synthesis, we paid special attention to regions known to contain antigenic reactivity, such as the C100-3, 5-1-1, and C33 regions (*14, 15*), or to regions with predicted but not yet confirmed antigenic reactivity, such as the entire NS5a and the C-terminal region of the NS5b. The number of peptides spanning different regions differed. In the NS3 protein, the region 1080–1313 aa was covered with 27 peptides, the region 1316–1456 aa

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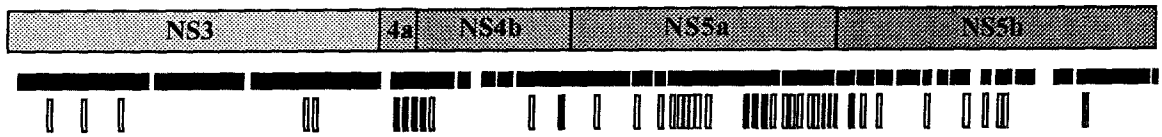

FIG. 1. Regions selected for the synthesis of peptides (shown by horizontal black bars) and the location of immunoreactive synthetic peptides (shown by small vertical bars) within the HCV nonstructural proteins NS3–NS4–NS5. Open vertical bars indicate weakly reactive peptides and closed vertical bars indicate relatively strong antigenically reactive peptides (for more detail, see Tables 1 and 2).

with 15 peptides, and the region 1478–1657 aa with 20 peptides. Two of the most thoroughly investigated regions of the NS4 protein are located at positions 1689–1789 aa and 1899–1980 aa and were spanned with 10 and 5 peptides, respectively. Since the entire NS5a is relatively hydrophilic as predicted by the method by Kyte and Doolittle (*34*) and composed of mainly random coil or β-turn structures as predicted by the method by Ptitsyn and Finkelstein (*35*), all of which are characteristics of antigenically active sites, this region was spanned with a large number of peptides. The region at position 1987–2083 aa was spanned with 8 peptides, the region at position 2150–2332 aa with 16 peptides, and the region at position 2336–2415 aa with 11 peptides. In the NS5b protein, a number of small scattered regions were selected for peptide synthesis. The most extended region covered with synthetic peptides is located at the C-terminus of the NS5b at position 2883–2990 aa. This region was spanned with 9 synthetic peptides.

Peptides were synthesized by FMOC chemistry (*36*) on an ACT Model MPS 350 multiple peptide synthesizer (Advanced Chemtech, Louisville, KY). After characterization by amino acid analysis, high performance liquid chromatography, and capillary electrophoresis, peptides were used directly in enzyme immunoassay as described elsewhere (*37*). Briefly, synthetic peptides were adsorbed to microtiter wells (Immulon II, Dynatech Labs, Inc.) at a concentration of 5 μg per well and allowed to interact with human anti-HCV sera diluted 1:50 for 1 hr at 37°. Antigenic activity of peptides was identified using affinity-purified antibodies to human IgG conjugated to horseradish peroxidase (Boehringer-Mannheim, Indianapolis, IN). Peptides were judged immunoreactive if the *P/N* ratio was greater than 3.0, where *P* represents the optical density (OD) value at 490 nm obtained with an anti-HCV-positive specimen and *N* represents the OD value of negative controls. A *P/N* ratio equal to 3 was equivalent to the mean OD values of negative control sera plus at least 3.5 standard deviations of the mean. Each peptide was individually analyzed at least three times with each of 20 anti-HCV-positive and 20 anti-HCV-negative sera. A cutoff was established individually for each peptide using all 20 anti-HCV-negative sera. It is important to note that among anti-HCV-negative sera, not a single specimen when analyzed with those peptides presented in Tables 1 and 2 exceeded the established cutoff value, indicating the absence of nonspecificity for these peptides. Typically, the OD value for negative controls was 0.02 ± 0.005. All serum samples were randomly selected from a collection of specimens reposited at the Hepatitis Branch, Centers for Disease Control and Prevention (Atlanta, GA). Anti-HCV-negative sera were obtained from normal blood donors. Anti-HCV-positive sera were collected from chronically HCV-infected individuals. The anti-HCV status of all sera was identified with the second generation anti-HCV EIA (Abbott Laboratories, Chicago, IL) and confirmed with a semiautomated dot blot immunoassay (Abbott MATRIX, Abbott Laboratories, Chicago, IL).

The results of the analysis of the anti-HCV reactivity of synthetic peptides are shown in Fig. 1 and Tables 1 and 2. Despite the recognized strong antigenic reactivity of the NS3 protein, which has been confirmed by many investigators, we were unable to identify any broadly reactive peptides among the 62 peptides analyzed from this region. Five peptides from this region were identified as marginally reactive. These peptides reacted with only one or two anti-HCV-positive sera (Table 1). This result suggests that the NS3 antigenic epitopes can be efficiently modeled only by large recombinant proteins and are poorly mimicked within relatively short synthetic peptides. Epitopes located within the NS3 protein seem to be conformationally dependent. Of five immunoreactive peptides from the NS3 protein, only two peptides, 60 and 117, are significantly overlapped (Table 1) and may share an antigenic epitope. Therefore, these data suggest the presence of four antigenic epitopes that may be modeled with synthetic peptides within the NS3 protein. However, there may be more than four antigenic sites within the NS3 region since some immunodominant epitopes within this region may have been neglected in our analysis. It is well known that synthetic peptides fail to reproduce topographic (discontinuous) antigenic epitopes, which compose in some cases more than 98% of all antigenic epitopes of a protein (*38*). Synthetic peptides of different sizes should be used to examine the fine antigenic structure of the NS3 region. A recent study on the antigenic reactivity of two peptides derived from the NS3 protein demonstrated the existence of at least one additional epitope at position 1479–1497 aa (*28*). A synthetic peptide with a similar aa sequence (1478–1497 aa) was analyzed in our study. This peptide, however, did not demonstrate immunoreactivity. This discrepancy may be due to differences in sequences between HCV strains used for the synthesis of peptides or in the sera used for the analysis.

TABLE 1

IMMUNOREACTIVE PEPTIDES FROM THE NS3 AND NS4 PROTEINS

Region	Peptide	Location, aa	Sequence	Activity[a]	*P/N*[b]
NS3	99	1126–1145	GSSDLYLVTRHADVIPVRRR	1/20	19.4
	90	1181–1200	RAAVCTRGVAKAVDFIPVEN	1/20	15.6
	107	1254–1273	LNPSVAATLGFGAYMSKAHG	2/20	3.5; 5.5
	117	1563–1582	TGLTHIDAHFLSQTKQSGEN	1/20	18.8
	60	1569–1588	DAHFLSQTKQSGENLPYLVA	2/20	21.0; 24.5
NS4	50	1689–1708	SGKPAIIPDREVLYREFDEM	8/20	21.8 (9.8–34.9)
	51	1691–1710	KPAIIPDREVLYREFDEMEE	9/20	17.3 (5.8–29.9)
	52	1701–1720	LYREFDEMEECSQHLPYIEQ	5/20	13.3 (4.7–22.8)
	53	1711–1730	CSQHCPYIEQGMMLAEQFKQ	9/20	12.6 (5.1–35.4)
	54	1721–1740	GMMLAEQFKQKALGLLQTAS	1/20	9.1
	57	1864–1885	VAFKIMSGEVPSTEDLVNLL	1/20	21.7
	59	1921–1940	AFASRGNHVSPTHYVPESDA	18/20	40.0 (5.3–85.5)

[a] The number of sera immunoreactive with synthetic peptides of 20 anti-HCV-positive serum specimens.

[b] For peptides reactive with two specimens, *P/N* is shown for both sera; for peptides reactive with more than two specimens, the mean of the *P/N* ratio for all reactive sera is presented with the range of *P/N* values shown in parentheses.

TABLE 2

IMMUNOREACTIVE PEPTIDES FROM THE NS5 PROTEIN

Peptide	Location, aa	Sequence	Activity[a]	*P/N*[b]
212	2032–2051	GAQITGHVKNGSMRIVGPKT	1/20	4.3
216	2085–2103	VAAEEYVEVTRVGDFHYVT	2/20	5.2; 5.5
218	2126–2145	DGVRLHRYAPVCKPLLREEV	1/20	4.4
220	2157–2175	GSQLPCEPEPDVAVLTSML	4/20	17.5 (13.0–26.6)
221	2169–2188	AVLTSMLTDPSHITAETAKR	2/20	12.3; 19.9
222	2182–2200	TAETAKRRLARGSPPSLAS	1/20	5.3
223	2190–2208	LARGSPPSLASSSASQLSA	2/20	10.9; 19.1
224	2202–2221	SASQLSAPSLKATCTTHHDS	1/20	6.3
226	2220–2238	DSPDADLIEANLLWRQEMG	1/20	4.1
231	2271–2290	PAEILRKPRKFPPALPIWAR	8/20	44.9 (5.0–114.7)
232	2279–2298	RKFPPALPIWARPDYNPPLL	4/20	31.5 (11.8–81.0)
233	2295–2313	PPLLESWKDPDYVPPVVHG	10/20	24.9 (4.1–98.4)
234	2313–2332	GCPLPSTKAPPIPPPRRKRT	3/20	30.6 (11.6–49.0)
235	2336–2354	TESTVSSALAELATKTFGS	2/20	12.8; 16.0
236	2346–2365	ELATKTFGSSGSSAVDSGTA	1/20	10.1
237	2353–2372	GSSGSSAVDSGTATGPPDQA	2/20	4.7; 5.9
240	2374–2392	DDGDKGSDVESYSSMPPLE	1/20	15.9
241	2377–2396	DKGSDVESYSSMPPLEGEPG	1/20	17.9
242	2381–2400	DVESYSSMPPLEGEPGDPDL	8.20	14.0 (4.0–34.7)
243	2385–2404	YSSMPPLEGEPGDPDLSDGS	8/20	13.9 (3.4–40.8)
244	2390–2409	PLEGEPGDPDLSDGSWSTVS	6/20	13.4 (4.2–31.0)
247	2434–2452	AAEESKLPINPLSNSLLRH	7/20	11.4 (3.7–31.7)
248	2460–2479	TSRSASLRQKKVTFDRLQVL	1/20	6
250	2505–2523	EEACKLTPPHSAKSKFGYG	1/20	22.9
255	2607–2626	GPSYGFQYSPGQRVEFLVNT	1/20	4.5
258	2681–2699	VGGPLTNSKGQNCGYRRCR	1/20	8.1
259	2717–2735	KATAACRAAKLQDCTMLVN	4/20	11.6 (4.4–17.9)
260	2744–2763	ESAGTQEDAAALRAFTEAMT	1/20	8.1
261	2760–2778	EAMTRYSAPPGDPPQPEYD	1/20	8.9
267	2894–2913	HSYSPGEINRVASCLRKLGV	7/20	12.9 (6.4–33.6)

[a,b] See the legend to Table 1.

TABLE 3

IMMUNOREACTIVITY OF SYNTHETIC PEPTIDES FROM THE NS3 AND NS4 PROTEINS WITH INDIVIDUAL SERA

		Serum specimens																			
Region	Peptide	65	66	67	68	71	72	73	74	75	76	77	78	80	81	83	84	85	86	87	88
NS3	99	C																			
	90														C						
	107							B													A
	117		C																		
	60		D														D				
NS4	50				C				E	C					D		D	E		C	B
	51				B				D	B				C	D		D	D		C	B
	52				B				C					A	C			D			
	53	B				B			A	C	B	B	E					B			E
	54																				B
	57		D																		
	59	F	B	C	D	E		B	D	F	E	F	F	F	B	D	F	B		C	F

Note. A, *P/N* = 3.0–4.9; B, *P/N* = 5.0–9.9; C, *P/N* = 10.0–19.9; D, *P/N* = 20.0–29.9; E, *P/N* = 30.0–49.9; F, *P/N* > 50.0.

In terms of antigenic reactivity modeled with synthetic peptides, the NS4 protein is much more reactive than the NS3 protein. Of 20 synthetic peptides examined, 7 demonstrated strong immunoreactivity (Table 1). Five of these, peptides 50, 51, 52, 53, and 54, are located in the 5-1-1 region which has been shown to contain strong antigenic reactivity (*14*). Since peptides 50 and 53 are not overlapped (Table 1), the 5-1-1 region contains at least two linear epitopes. After analyzing the sequences of these peptides and the pattern of reactivity with different anti-HCV-positive sera (Table 3), we conclude that peptides 50, 51, and 52 share one antigenic epitope and peptides 53 and 54 share another epitope, and the common region within each of these peptide groups may represent the actual antigenic epitope or an essential element of the epitope. Thus, the 5-1-1 region may contain two epitopes, one located at position 1701–1708 aa with the sequence LYREFDEM, and the other located at position 1721–1730 aa with the sequence GMMLAEQFKQ. Similarly, two antigenic epitopes were recently found in the 5-1-1 region using monoclonal antibodies and synthetic peptides (*31, 33*).

In addition to epitopes in the 5-1-1 region, we found two more immunoreactive peptides in the NS4 protein (Tables 1 and 3). One marginally reactive peptide, 57, is positioned inside the C100-3 region. Another peptide, 59, containing the most broadly reactive antigenic epitope found in this study, overlaps with the C-terminal region of C100-3. To analyze this antigenic region in more detail, we synthesized an additional small set of nine peptides spanning the sequence of the NS4 protein at position 1905–1955 aa (Fig. 2). These peptides were examined

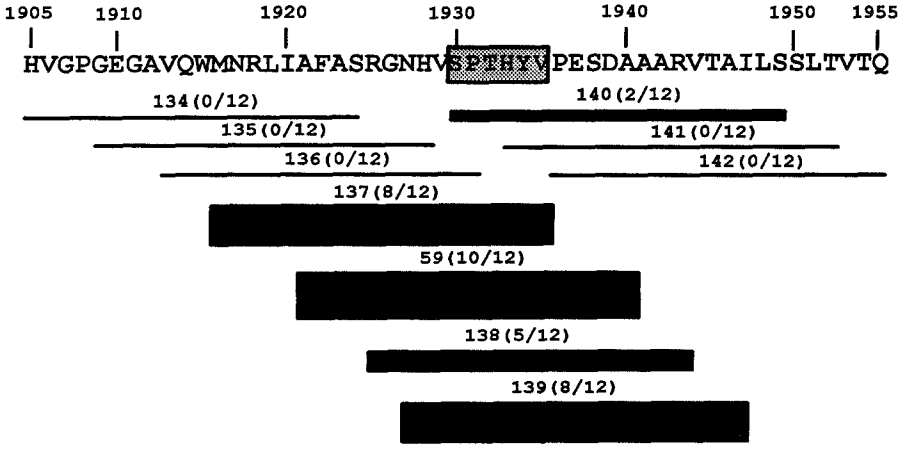

FIG. 2. Identification of the antigenic epitope located within peptide 59. Primary structure of the HCV polyprotein region at position 1905–1955 aa (*4*) and the amino acid sequence of synthetic peptides 59 and 134–142 are shown. The degree of immunoreactivity is indicated by the thickness of the horizontal bars. The numbers in parentheses show how many immunoreactive serum specimens were found for each peptide of 12 anti-HCV-positive sera analyzed. The sequence common for all immunoreactive peptides identified within this region is framed and shadowed.

TABLE 4

REACTIVITY OF SYNTHETIC PEPTIDES FROM THE NS5 PROTEIN WITH INDIVIDUAL SERA

	Serum specimens																			
Peptide	65	66	67	68	71	72	73	74	75	76	77	78	80	81	83	84	85	86	87	88
212		A																		
216	B																		B	
218																A				
220					C			D	C							C				
221	C								C											
222																				B
223	C														C					
224	B																			
226									A											
231	F			E	F						C	B			D	F				E
232				C	C											F				C
233	A			E	D				BTABLE 1		A	E			C	F	A			C
234												E				E				C
235									C			C								
236												C								
237																B				A
240									C											
241									C											
242	A				B		B		D			B			B	E				C
243	A				B		C		D			B			A	E				C
244	B				B				D			A				E				B
247	B			B	C				B						A	E				B
248																B				
250														D						
255			A																	
258																	B			
259			C		B										A		C			
260									B											
261																			B	
267	B			B	C		B		B							E				C

Note. A, *P/N* = 3.0–4.9; B, *P/N* = 5.0–9.9; C, *P/N* = 10.0–19.9; D, *P/N* = 20.0–29.9; E, *P/N* = 30.0–49.9; F, *P/N* > 50.0.

for antigenic reactivity with 12 anti-HCV-positive sera selected from the panel used in this study. Peptides 137, 138, 139, and 140 were immunoreactive in addition to peptide 59 (Fig. 2). The only common region shared by these immunoreactive peptides is the sequence SPTHYV located at position 1930–1935 aa. Therefore, this region appears to be the antigenic epitope located within peptide 59, which is located outside of the C100-3 region. Thus, we found at least four antigenic epitopes within the NS4 protein with 1 epitope located within NS4a (Fig. 1 and Table 1; peptides 50, 51, and 52) and three epitopes within NS4b (Fig. 1 and Table 1; one within peptides 53 and 54, one within peptide 57, and one within peptide 59).

The results of the analysis of the antigenic properties of 68 synthetic peptides prepared from the NS5 protein are shown in Tables 2 and 4. We found 30 peptides demonstrating HCV-specific immunoreactivity, representing almost 45% of all NS5 peptides examined in this study. However, 19 peptides reacted with only one or two anti-HCV-positive sera (Tables 2 and 4). Peptides 212, 218, 226, and 255 reproducibly bound an antibody from different single specimens with consistently low *P/N* values equal to 4.1–4.5. We did not find any examples of reactivity to these peptides with anti-HCV-negative sera and, therefore, considered these peptides together with other weakly reactive peptides as marginally immunoreactive. Very few publications describe epitopes within the HCV NS5 protein. Two antigen epitopes have been recently identified by Yuki *et al.* (*28*). One epitope was found at position 2373–2386 aa and the second epitope was found at position 2467–2486 aa. Both of these epitopes were also identified in our study as marginally reactive (Table 2, peptides 240 and 248).

Recently, an additional protease cleavage site was identified within the HCV NS5 protein, dividing it into halves, with the N-terminal half named NS5a of unknown function(s) and the C-terminal half named NS5b and possibly representing the RNA-dependent RNA polymerase (*39*). Twenty-one immunoreactive peptides (70% of all immunoreactive NS5 peptides) are located within the NS5a protein (Table 2, peptides 212–244), while only 9

peptides (30% of all immunoreactive NS5 peptides) are located within the NS5b (Table 2, peptides 247–267). In total, 38 peptides were prepared from NS5a and 30 peptides from the NS5b. Therefore, without regard to the breadth of immunoreactivity, 55% of peptides prepared from NS5a and 30% of peptides prepared from NS5b contain linear antigenic epitopes modeled with short peptides, indicating that the NS5a region is very immunoreactive. For comparison, only 7.5% of the NS3 peptides and 35% of the NS4 peptides had HCV-specific antigenic reactivity. Taking into consideration the location of peptides and the pattern of reactivity of these peptides with different anti-HCV sera (Table 4), 24 B-cell epitopes may be identified within the NS5 protein, with 15 epitopes located within NS5a and 9 epitopes within NS5b. Each NS5b immunoreactive synthetic peptide seems to have its own antigenic epitope (Table 2). Within NS5a, however, peptides 223 and 224; 231 and 232; 235 and 236; 240 and 241; and 242, 243, and 244 appear to share B-cell epitopes within each group (Table 2). The remaining 10 epitopes are located within individual peptides (Table 2). In the NS5a protein, four antigenic epitopes were reactive with 25–50% of anti-HCV-positive sera (Table 2); the first epitope is located within peptide 220, the second is shared by peptides 231 and 232, the third is represented within peptide 233, and the fourth is shared by overlapped peptides 242, 243, and 244. The NS5b region has three strong epitopes (Table 2; peptide 247, 259, 267). Collectively, peptides containing these strong broadly reactive epitopes interact with 16 of 20 anti-HCV-positive sera used in this study. The two sera that did not react with NS3 and NS4 synthetic peptides also did not react with any peptide derived from NS5 protein (Tables 3 and 4). Nonetheless, antigenic epitopes located within the NS5 protein frequently elicit antibodies during the course of HCV infection, suggesting that these epitopes may have significant value as diagnostic reagents for the development of HCV-specific tests.

Finally, significant variation in the primary structure of the HCV proteins has been reported (*4–13*). Obviously, sequence variation may affect the immunoreactivity of many of the antigenic epitopes identified in this study. For a more complete understanding of the antigenic organization of the HCV polyprotein, peptides derived from different HCV strains should be prepared and analyzed. Such an analysis is currently under investigation.

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