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Isotype-specific immune response to a single hepatitis C virus core epitope defined by a human monoclonal antibody: diagnostic value and correlation to PCR

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Summary In this study we tested the seroreactivity of 223 selected anti-HCV-reactive blood donors to the human B-cell epitope N-VYLLPR-C (C_{34-39}) of the hepatitis C virus core antigen. The epitope was recently identified and characterized by the human monoclonal IgG antibody Ul/F10 [23] and is located within the amino acid residues 34–39 of the aminoterminal core region. The blood donor sera were selected from anti-HCV ELISA (Ortho, 2nd generation)-reactive samples. Sixty-seven of these sera were further reactive in RIBA (Ortho, 2nd generation). According to their RIBA pattern, these samples were divided into four groups. Samples in the first group ($n=18$) reacted to all four recombinant HCV antigens. The samples of the second ($n=9$) and third group ($n=8$) reacted to c22-3/c33c and c22-3/c100-3, respectively. Sera from group 4 ($n=32$) showed a RIBA indeterminate pattern with reactivity only to c22-3. All 223 samples were analyzed for anti-C_{34-39} antibodies by ELISA, and the 67 RIBA-reactive samples were additionally tested for the presence of HCV RNA by RT/PCR. In groups 1 and 2, over 80% of the samples showed anti-C_{34-39} reactivity which was restricted to the IgG_1 isotype. In contrast, in groups 3 and 4, antibodies to the epitope C_{34-39} were detected in less than 10% of the samples. Interestingly, the anti-C_{34-39} response correlates with the presence of HCV RNA; 95.5% of the samples had coincident results in all subgroups. None of the RIBA-negative sera showed a specific seroreaction to the C_{34-39} peptide.

Key words Hepatitis C virus · Human monoclonal antibody · Epitope · Immune response

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Introduction

Hepatitis C virus (HCV) has been identified as the major agent of post-transfusion non-A, non-B hepatitis. The virus is found to be closely related to the Flavi- and Pestiviruses and is classified as a third genus in the family of Flaviviridae [17]. The recent cloning of the genome and the subsequent development of serological assays based on recombinant proteins have greatly improved the diagnosis of HCV infections in human beings [7, 8, 18].

The first available diagnostic test included the recombinant protein c100-3 from the nonstructural NS4 domain as a solid-phase antigen for the detection of anti-HCV antibodies [18]. Subsequent studies showed that anti-c100 is not an efficient marker for diagnosis of HCV infection, and consequently the assay lacked sensitivity and specificity [10, 14]. The incorporation of recombinant proteins from the highly conserved structural core region (c22-3) and from the nonstructural NS3 region (c33c) in the ELISA increases the sensitivity and specificity for the identification of infected individuals. Several data indicate that antibody response to c22 develops rapidly after the onset of infection [5, 16, 30]. The analysis of the antibody response with recombinant core fragments and synthetic peptides resulted in the identification of major immunodominant regions. These are located predominantly in the aminoterminus of the core protein [6, 20, 22].

At present, the recombinant immunoblot RIBA is available as a supplementary antibody test to evaluate positive ELISA results [19, 24, 25]. An advantage of this assay is that antibody reactivity can be related to single antigen bands on the test membrane. Therefore, this test increases the antibody specificity by offering different conditions than those of microtiter plates. Yet it is not a true confirmatory test for HCV infection because it includes the same antigens as those used in the ELISA. The pattern of antibody response to HCV proteins seen by RIBA analysis is very variable in individual cases. In addition, some anti-HCV ELISA-positive

sera show an indeterminate RIBA pattern with reactivity to only one of the test bands. The biological and clinical significance of such a pattern is unknown.

A highly sensitive, antibody-independent technique for the diagnosis of HCV infection is the reverse transcription/polymerase chain reaction (RT/PCR) which allows the direct detection of viral genome sequences in serum/plasma and hence the determination of potential infectivity [9, 27].

We have recently identified the linear epitope N-VYLLPR-C (C_{34-39}) within the HCV core protein as a target for the human immune response to HCV infection [23]. The epitope is located within the immunodominant aminoterminus [19–22]. In this study, we were interested in analyzing the antibody response of anti-HCV-reactive blood donors to this epitope. Furthermore, the relationship between the presence of anti-C_{34-39} antibodies and viral RNA was investigated.

Materials and methods

Blood donors

Sera from 223 blood donors anti-HCV reactive in ELISA (Ortho, 2nd generation, Neckargemünd, FRG) and with different patterns in RIBA (Ortho, 2nd generation) were selected for the anti-C_{34-39} ELISA. The RIBA-reactive samples ($n=67$) were divided into four groups according to their pattern: group 1 ($n=18$), seroreactive to c22-3, c33c, c100-3, and 5-1-1; group 2 ($n=9$), seroreactive to c22-3 and c33c; group 3 ($n=8$), seroreactive to c22-c and c100-3; group 4 ($n=32$), seroreactive to c22-3 (RIBA indeterminate). Elevated ALT values (two times the upper limit of 35 U/l) were found in only one sample from group 2. Sera from 25 healthy anti-HCV-negative blood donors were collected for negative control.

Anti-C_{34-39} ELISA

The ELISA for the detection of antibodies to the epitope C_{34-39} defined by the human monoclonal antibody Ul/F10 was performed with a synthetic peptide (VYLLPRRC) covalently linked to maleimide-activated bovine serum albumin (BSA) (Pierce, Nürnberg, FRG). The conjugate was coated onto PVC microtiter plates (Falcon, Microtest III) at a concentration of 0.5 µg/ml in PBS. To evaluate the specificity of the ELISA, negative control wells were coated with an irrelevant BSA conjugate (NWSPTAC-BSA). The coating was complete after overnight incubation at 4° C. Plates were then blocked by the addition of 2% BSA in PBS for 2 h at RT.

Serum dilutions were added after three washes with PBS containing 0.1% Tween-20 and incubated for 2 h at RT. All sera were assayed at a dilution of 1:50 in PBS containing 2% BSA and 1% Tween-20. Plates were then washed again three times and bound antibodies were detected with biotinylated goat anti-human IgG $F(ab)_2$ fragments (Dianova, Hamburg, FRG) 1:10000 diluted in PBS with 0.2% BSA and 0.1% Tween-20. The incubation was performed for 1 h at RT; it was followed by a 30 min incubation with streptavidin/peroxidase conjugate diluted 1:2000 (Dianova). After six washes antibody binding was visualized by the OPD reaction. Plates were incubated for 10 min in the dark and the reaction was stopped by adding 2 N sulfuric acid. Optical densities (OD) were measured at 492 nm on a microplate ELISA reader.

A competitive inhibition test was performed in order to evaluate the specific binding of the human monoclonal antibody Ul/F10 to microtiter plate immobilized C_{34-39}-BSA. For this purpose the monoclonal antibody was preincubated with the soluble peptide for 2 h at RT.

To determine the IgG isotype of the anti-C_{34-39} response, positive serum samples were distributed to four wells of a microtiter plate and analyzed with biotin-conjugated mouse monoclonal antibodies specific for human IgG subclasses (Sigma). Biotin residues were detected as described for anti-human IgG.

The cutoff value of the anti-C_{34-39} ELISA was calculated by multiplying the mean absorbance of 25 anti-HCV seronegative healthy blood donors by a factor of 3. To discriminate false-positive sera depending on unspecific binding to microtiter plates, the OD values of C_{34-39} coated wells were additionally compared with those of negative control wells.

PCR analysis

RT/PCR RNA extraction, cDNA synthesis, and PCR were carried out as described previously [3]. Serum samples were tested with two sets of primers: the first one codes for sequences in the 5′ noncoding region and the second for sequences in the putative capsid region of the HCV genome. Samples were considered positive when they produced at least one positive result out of two amplifications [2].

Results

To evaluate the specificity of the anti-C_{34-39} ELISA, a blocking experiment was performed using soluble C_{34-39} peptide as a competitor molecule. As shown in Fig. 1, the binding of the human monoclonal antibody Ul/F10 was inhibited in a concentration-dependent manner by preincubation with soluble peptide.

Anti-HCV ELISA-reactive but RIBA-negative blood donor sera were tested for anti-C_{34-39} antibodies. None of these 156 sera showed a specific reaction in the anti-peptide ELISA. Five samples showed an enhanced OD in the C_{34-39}-BSA plates, as well as in the NWSPTAC-BSA-coated plates. For this reason, these samples were classified as false positive (Table 1).

Further, blood donor samples from group 1, positive in RIBA to all four recombinant antigens, were tested by the anti-C_{34-39} ELISA. The results of this assay and those from the RT/PCR are listed in Table 2. Sixteen of

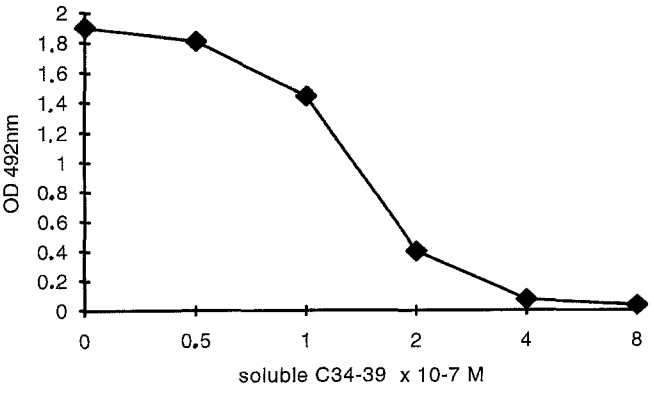

Fig. 1 Dose-dependent blocking of Ul/F10 binding to solid-phase C_{34-39} preincubation with soluble peptide for 2 h at RT

Table 1 OD values of the false-positive anti-C_{34-39} sera in comparison to the reactivity of the monoclonal antibody Ul/F10. Five of the 156 tested anti-HCV ELISA-positive and RIBA-negative sera reacted unspecifically in the peptide assay

Donor no.	OD_{492}[a] C_{34-39} coated	Control peptide
12	0.497	0.510
19	0.210	0.235
48	0.180	0.175
96	0.276	0.341
102	0.159	0.185
Ul/F10	1.980	0.016

[a] Cutoff OD_{492} value 0.150

Table 2 Detection of anti-C_{34-39} and HCV RNA in samples from donor group 1: RIBA positive to all four recombinant antigens ($n=18$)

	Anti-C_{34-39}	
	+	−
HCV RNA +	13	−
HCV RNA −	3	2

Table 3 Detection of anti-C_{34-39} and HCV RNA in samples from donor group 2: RIBA positive to c22-3/c33c ($n=9$)

Donor no.	OD_{492}[a] Anti-C_{34-39}	HCV RNA
175	1.63	+
176	0.90	+
177	0.40	+
178	1.10	+
179	1.62	+
180	1.11	+
181	0.08	−
182	0.10	−
183	1.20	+

[a] Cutoff OD_{492} value 0.150

18 samples (89%) in this group were positive for anti-C_{34-39}. In 13 of these anti-peptide-positive samples HCV RNA was detectable. The two anti-peptide negative sera were also negative for HCV RNA amplification.

In group 2 the percentage of anti-C_{34-39}-reactive samples attained was comparable to the percentage in group 1, although we disposed of a limited number of samples. As shown in Table 3, seven of nine RIBA-positive donors, showing reactivity to c22 and c33c, developed an anti-C_{34-39} response. HCV RNA amplification turned out to be positive for the same seven sera. The remaining two samples were negative with both assays.

None of the eight serum samples in group 3 had a positive result, neither in the anti-peptide nor in the RT/PCR assay, as summarized in Table 4. These samples had been evaluated as positive by RIBA, showing reactivity to c22 and c100.

Table 4 Detection of anti-C_{34-39} and HCV RNA in samples from donor group 3: RIBA positive to c22-3/c100-3 ($n=8$)

	Anti-C_{34-39}	
	+	−
HCV RNA +	−	−
HCV RNA −	−	8

Table 5 Detection of anti-C_{34-39} and HCV RNA in RIBA-indeterminate sera reactive to c22 (donor group 2, $n=32$)

	Anti-C_{34-39}	
	+	−
HCV RNA +	3	−
HCV RNA −	−	29

Table 6 Relationship between the presence of anti-C_{34-39} and HCV RNA in blood donor samples independent of the investigated subgroups ($n=67$)

	Anti-C_{34-39}	
	+	−
HCV RNA +	23	−
HCV RNA −	3	41

Table 7 IgG subclass distribution of the anti-C_{34-39} immune response

No. of positive sera	IgG subclass reactivity			
	γ_1	γ_2	γ_3	γ_4
27	27	−	−	−

Group 4 consisted of serum samples reactive only to c22 in RIBA. This reaction pattern is termed "RIBA indeterminate". Three of 32 samples were reactive for anti-C_{34-39}. As shown in Table 5, HCV RNA was detectable in these three anti-peptide-positive samples.

Finally, considering all 67 RIBA-reactive samples, independent of the subgroups, 95.5% coincident results were obtained by the anti-C_{34-39} and the RT/PCR assay, as summarized in Table 6. The three discordant sera were positive for anti-C_{34-39} antibodies but had no detectable virus RNA.

In order to identify the isotype of the anti-C_{34-39} immune response, all positive sera were additionally tested with subclass-specific antibodies to human IgG_1–IgG_4. The samples were incubated on immobilized peptide in quadruple sets and analyzed individually with subclass-specific antibodies. As summarized in Table 7, the antibody reactivity was restricted to γ_1. No reactivity was observed with subclass-specific anti-γ_2–γ_4 antibodies.

Discussion

The core region of HCV contains highly conserved immunodominant, epitopes that elicit antibody response early in HCV infection. From serological studies with small core fragments, it is known that the aminoterminus is very immunogenic [20–22]. We have recently identified a single B-cell epitope within this region. The epitope is defined by the human monoclonal IgG_1 antibody UI/F10, isolated from an HCV-infected blood donor. With this epitope, we developed an ELISA for the detection of corresponding antibodies in sera from blood donors and patients.

The evidence that in the RIBA-positive group 1 a high rate of reactivity (89%) to the peptide C_{34-39} has been reached, suggests that this epitope is important in the humoral immune response to HCV. Ferroni et al. [12] evaluated immunoreactivities of 15-mer peptides from the core region which were selected by an antigenic algorithm. The most immunogenic peptide from this study includes the epitope C_{34-39}, and antibodies were detected in 44 of 45 RIBA-confirmed positive blood donors. Additional data on the significance of this core region come from Akatsuka et al. [1] and Cerino et al. [4]. Both groups have isolated human monoclonal anti-core antibodies reactive with the fragments $core_{33-42}$ and $core_{34-45}$, respectively. As demonstrated in our study, the IgG subclass distribution of the anti-C_{34-39} response seems to be restricted to γ_1, a common subclass for anti-virus antibodies [26]. In contrast to the results in group 1, samples which are considered RIBA indeterminate due to reactivity only to c22 (group 4) were not reactive in the peptide assay in most cases. From these 32 samples only three contained anti-C_{34-39} antibodies (9%).

All RIBA-reactive samples were investigated by PCR. HCV RNA was detected in 89% of the anti-C_{34-39}-positive samples from groups 1–3. None of the anti-peptide-negative samples were positive in the PCR assay. In group 4, HCV RNA was detectable in all three anti-C_{34-39}-positive samples. The presence of virus RNA in serum is a marker for infectivity; however, RT/PCR may fail in a few cases, due to a very low titer of virus in the peripheral blood. Yet in this study, RT/PCR and anti-C_{34-39} ELISA reached over 95% coincident results, indicating that the performance of the two assays was equally sensitive and specific. Moreover, our results are in agreement with recent studies which suggest that only a small fraction of donors with antibodies to c22 are, in fact, viremic [3, 13, 31].

The majority of the 29 RIBA-indeterminate samples negative for anti-C_{34-39} and HCV RNA possibly reacted unspecifically to c22 in the commercial ELISA and RIBA. The recombinant core protein introduced in these assays may contain epitopes for cross-reacting antibodies not associated with HCV. Eliminating such sequences is a major feature in the development of more specific anti-HCV assays. Therefore, the identification of specific immunodominant epitopes with human monoclonal antibodies may be useful. Such characterized epitopes can substitute recombinant core protein to maximize specificity. By comparing the OD values of C_{34-39}-coated wells with negative control wells we additionally discriminated sera which bound unspecifically to the assay plates. In this study such unspecific reactivity was found in only five of the 156 ELISA-reactive but RIBA-negative sera (Table 1).

Noteworthy is the fact that the anti-peptide analysis provided extremely different results in groups 2 and 3. Samples in these groups were considered RIBA-positive, with reactivity to two recombinant antigens. Whereas in group 3 we did not find any sample positive to C_{34-39}, seven of nine (83%) samples in group 2 were seroreactive. PCR results were 100% coincident with the peptide assay in both groups. To evaluate the meaning of this discrepancy, larger groups must be investigated. Possibly, there are more false-positive reactions related to c22 in combination with c100 than c22 in combination with c33. From early studies with first-generation tests based only on c100, it is known that this antigen tends to produce unspecific reactions [14]. Our results indicate that c33 might be a better marker than c100. It has also been reported that anti-c33 often appears in combination with c22 in early acute infection [28, 29].

An important result in this study is the high rate of concordance for anti-C_{34-39} antibodies to HCV viremia detected by PCR. This finding is independent of the subgroup investigated. Sixty-four of 67 tested blood donor samples had coincident results (95.5%). The remaining three discordant serum samples were found in group 1. They were both RIBA and peptide assay positive, but HCV RNA negative. It has been suggested that the presence of anti-HCV-positive but PCR-negative seroreactions may represent a past self-limiting infection [11]. In these individuals the immunological marker may persist for some time after the virus has been eliminated, or maybe restricted to the liver tissue with no detectable RNA in plasma [15].

In conclusion, if we consider the PCR a standard method for the detection of infectivity, screening of blood donors with the C_{34-39} epitope appears to be more closely related to the presence of HCV RNA than screening with the recombinant core protein used in the present serological tests. Therefore an improvement in the specificity of the RIBA-2 might be attained through the replacement of the recombinant core proteins by synthetic immunoreactive peptides. Identification of B-cell epitopes with human monoclonal antibodies and their subsequent characterization may help to select such specific immundominant epitopes.

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