Genotype Dependence of Peptide-Based Immunoassays For the Detection of HCV Core Antibodies

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INTRODUCTION

Detection of hepatitis C virus (HCV) antibodies is partially influenced by the genotype of the infecting isolate. Immunoassays using genotype-1a-derived recombinants or peptides results in diminished reactivity among individuals infected with heterologous genotypes. We examined the magnitude of this effect on detection of core antibodies by using genotype-1a-derived core peptide immunoassays to test 254 HCV anti-core-positive individuals infected with genotypes 1–4 or 6. Peptides corresponding to amino acids 1–18, 10–24, and 11–28 reacted with 60%, 89%, and 85% of all samples, respectively. Peptide 1–18 detected 78% of individuals infected with genotype-1 or 2 but only 43% of those infected with genotypes 3, 4, or 6. Genotype-dependent reactivity was also observed for peptides 10–24 and 11–28. The use of a 34-mer peptide (encompassing amino acids 10–43) within the immunodominant region detected antibodies in 100% of specimens, thereby eliminating the genotype-dependent antibody detection observed with shorter peptides. Sequence differences between peptides and core of the infecting isolate did not entirely account for the genotype-dependent reactivity since some individuals displayed reactivity to peptides containing up to seven amino acid differences relative to the sequence of the infecting isolate, while others with identical core sequences had little or no reactivity. Thus, HCV core sequence divergence accounts for only a portion of the differential core antibody detectability observed when non-type-specific peptides are used. Differences in immune response between individuals infected with identical isolates also plays a significant role in core antibody detection using short peptides. J. Med. Virol. **80**:411–418, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: nucleocapsid; sequence divergence; immunodominant

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chronic HCV cases [Hino, 1994] and in nearly 100% of the acute cases by 12 weeks post-infection [Alter, 1992]. The second-generation assays showed a marked improvement over the first generation test in identifying HCV-infected blood donors. The third generation tests added a recombinant NS5 region protein, however, some studies indicated only a slight improvement in sensitivity of the third generation test over the second-generation tests [Courouce et al., 1994; Lee et al., 1995]. This improvement was largely attributed to changes in the NS3 protein rather than the inclusion of NS5 [Courouce et al., 1994].

The NS4 antigen utilized in the first generation assay has variable amino acid sequences across HCV genotypes, leading to inadequate detection of antibodies in HCV samples other than genotype-1. The type-specific antibody response to NS4 protein, in fact, has been utilized as a means to serotype HCV infections [Simmonds et al., 1993]. Although NS3 and core are more highly conserved, the antibody responses to genotype-specific antigens are higher among individuals infected with a homologous genotype. For example, Neville et al. [1997] demonstrated that immunoreactivity of type 1b and 4a-infected individuals was much higher when using type-homologous antigens compared to type-non-homologous antigens. However, the reduced signals are not sufficient to produce false negative results with the genotype-1a antigens utilized in the commercial antibody tests. Other groups have demonstrated less-than-optimal detection of antibodies when these assays are applied to individuals infected with non-type-1 isolates [Callahan et al., 1993; Nagayama et al., 1993; Dhaliwal et al., 1996].

Similar results have been reported for confirmatory assays [Halfon et al., 2002] including the RIBA strip immunoblot assay. For example, Zein et al. [1997] demonstrated that RIBA-2 anti-core indeterminates are more likely to occur among individuals infected with non-type-1 isolates. While the RIBA-2 assay utilized a recombinant core protein (c22-3) the third-generation test uses a peptide derived from the amino terminus of HCV core. Though the RIBA-3 test demonstrates better sensitivity than the RIBA-2 test [Tobler et al., 2000], there have been reports of differential reactivity between individuals infected with non-type-1 isolates. Dow et al. [1996] demonstrated that genotype-3, and to a lesser extent, genotype-2-infected individuals exhibit suboptimal reactivity toward the c100 and c33 bands in the RIBA-3 assay. Similarly, Beld et al. [1999] reported significantly higher RIBA-3 median antibody responses to core and NS4 among genotype-1-infected individuals compared to those infected with other genotypes.

Several reports have suggested that there is a genotype-specific reactivity to peptides of 30–50 residues derived from the amino-terminal region of core [Machida et al., 1992; Sato et al., 1994]. However, a comprehensive analysis of the genotype dependence of anti-core detection using peptides spanning the major epitope-containing region of HCV core has not been done. We sought to determine the genotype dependence of core antibody detection more closely by using a collection of 13 overlapping synthetic peptides derived from amino acids 1-130 of HCV core and testing a panel of 254 HCV antibody-positive individuals, as determined by EIA reactivity, where the infecting HCV genotype was known.

**MATERIALS AND METHODS**

**Human Sera**

HCV antibody-positive human serum and/or plasma were purchased from a commercial vendor (ProMedDx, Inc., Norton, MA) or were obtained from clinical laboratories in accordance with Institutional Review Board policies and procedures. Samples were identified only by code number. In some instances, the corresponding HCV genotype was provided; for all others, HCV genotype was determined via sequence analysis of the NS5b region (see below). All samples tested positive in an EIA using the recombinant core protein (amino acids 1–150) currently used in the Abbott HCV EIA second-generation blood donor-screening assay (data not shown).

**Coating of Peptides Onto Polystyrene Beads**

Peptides derived from the HCV-1 prototype genotype-1a isolate were synthesized by an in-house proprietary method or were custom synthesized through an outside vendor (Anaspec, Inc., San Jose, CA). Most peptides were synthesized with an amino terminal biotin residue that could be used to validate peptide coating onto the solid phase (see below). One quarter-inch polystyrene beads were used as the solid phase for the peptide EIAs. Prior to coating, beads were washed with 15% isopropanol at room temperature for 30 min without agitation. Isopropanol was removed and the beads were rinsed once with deionized water. The washed beads were then added to a vial containing the peptide diluted to 5 μg/ml in 0.1 M sodium phosphate buffer, pH 7.5 (0.233 ml per bead). Beads were incubated at 56°C for 2 hr with end-over-end mixing. Beads were then washed three times with PBS and then incubated in PBS containing 0.1% Triton X-100 at 40°C for 1 hr with end-over-end mixing. They were again washed three times in PBS and then incubated at 40°C in 5% BSA/PBS for 1 hr with end-over-end mixing. Beads were washed four times with PBS and then incubated at room temperature in PBS containing 5% sucrose without mixing for 20 min. Sucrose buffer was removed and beads were air dried. Coated beads were stored desiccated at 4°C.

**Bead-Coating Validation**

To determine whether the biotinylated peptides were actually coated onto the beads, an assay was performed in which beads were incubated in buffer containing horseradish peroxidase-labeled streptavidin (200–400 ng/ml). The beads were then washed with deionized water and substrate (0.3% O-phenylenediamine-2-HCl in citrate buffer containing 0.02% hydrogen peroxide) was added. After incubation for 30 min in...
the dark at room temperature, the reaction was quenched by the addition of 1 ml of 1N sulfuric acid and the OD at 492 nm measured.

Absorbance at 492 nm was directly proportional to the amount of peptide coated onto the bead surface.

**HCV Peptide EIAs**

Human sera from HCV antibody-positive individuals were tested with each of the peptide-coated beads as follows: serum was diluted 336-fold in sample diluent buffer (Tris buffer containing 20% goat serum, 10% calf serum, 0.2% Triton X-100, and sodium azide) of which 0.2 ml was added into a reaction well containing the peptide-coated bead and incubated at room temperature for 2 hr with mixing. Beads were then washed with deionized water followed by the addition of 0.2 ml of peroxidase-labeled goat anti-human IgG (1 ng/ml). Beads were incubated at room temperature for 60 min with mixing. Beads were washed with deionized water and then transferred into plastic tubes to which 0.3 ml of OPD (0.3% O-phenylenediamine-2-HCl in citrate buffer containing 0.02% hydrogen peroxide) substrate was added and incubated in the dark at room temperature for 30 min without mixing. The reaction was quenched by the addition of 1 ml of 1N sulfuric acid and the OD at 492 nm determined. The absorbance is directly proportional to the amount of antibody bound to the bead.

**Statistical Analysis**

Contingency tables were constructed for comparison of peptide EIA results for the group of individuals infected with HCV genotype-1 versus a group of individuals infected with another genotype. Hence, all comparisons were constructed in order to determine the accuracy of each EIA, which use only genotype-1 sequence-derived peptides, for detection of antibodies among genotype-1-infected compared to non-genotype-1-infected individuals. Positive and negative (i.e., below the provisional cutoff signal-to-negative ratio of 5.0) reactivity rates defined the categorical variables.

Statistical significance of EIA accuracy for detection of genotype-1-infected individuals versus those infected with another genotype was determined by using Fisher’s exact test (GraphPad Prism version 4.0, Windows, GraphPad Software, San Diego, CA) wherein a P-value of 0.05 or lower was considered significant.

**Sequencing and Sequence Analysis**

The amino acid sequence of the core gene from selected samples was determined via direct analysis of amplicons generated by RT-PCR using primers located within the 5′-untranslated region and at the 5′-end of the E1 gene. Sequencing was performed using ABI Model 377 Automated Sequencer and the ABI Big Dye Terminators as described by the manufacturer (Applied Biosystems, Foster City, CA). Sequences were compiled and analyzed using the Sequencher computer program (GeneCodes, Inc., Ann Arbor, MI) and aligned using the PILEUP program from the Wisconsin Package (version 10). HCV genotype was determined by sequence analysis of amplicons derived from a portion of the NS5b gene using PCR primers previously described [Simmonds et al., 1994]. Phylogenetic analysis was performed as previously described [Simmonds et al., 1994] by using a set of reference sequences for comparison. BLAST analysis of each sequence was performed against GenBank to validate the genotype determined by phylogenetic analysis. In most cases, the genotype of the HCV isolate with the highest similarity score matched that determined by the independent phylogenetic analysis.

**RESULTS**

**Peptide-Specific Immunoreactivity**

All human sera used in the study tested positive for HCV core antibodies by using an EIA that utilized an HCV genotype-1a core recombinant protein encoding amino acids 1–150 (data not shown). The samples were not selected for their anti-core reactivity, however, but for positivity in a licensed anti-HCV blood screening or diagnostic assay. The cohort of 254 individual specimens were comprised mostly of genotypes 1 (n = 94) and 3 (n = 107). Genotypes 2, 4, and 6 were represented by 28, 18, and 7 individuals, respectively. All specimens were tested in 13 different anti-core EIAs utilizing HCV core-derived peptides (Fig. 1 and Table I). The peptide allowing detection of antibodies in 100% individuals was that corresponding to amino acids 10–43. The assay with the next-highest sensitivity was that utilizing peptide 1–30 where 92.9% of specimens were reactive. The overlapping peptides 10–24 and 11–28 detected 89.4% and 84.6% of all individuals, respectively. The proportion of antibody positives detected decreased considerably for those EIAs utilizing peptides derived from sequences downstream of position 68 (Fig. 1). This is consistent with earlier reports that the immunodominant region of HCV core lies within the first 70 amino acids of the protein [Sallberg et al., 1992]. Reactivity toward either peptide 10–24 or 11–28 allowed detection of 244/254 (95.9%) of all specimens, a slightly higher percentage than that of peptide 1–30 alone. Among the 10 samples unreactive for peptides 11–28 or 10–24, 3 were not reactive in 12 of 13 peptide EIAs (all were reactive toward peptide 10–43) while the remainder were reactive to only one peptide (again excluding 10–43): 4 to peptide 1–30, and 3 to peptides downstream of amino acid 30. Furthermore, of the 10 non-11–28/10–28 reactive individuals, 9 were infected with genotypes 3, 4, or 6, and one with genotype-1b (reactive towards peptide 1–30). These results suggest a propensity for individuals infected with non-1-genotypes to exhibit lower reactivity to EIAs using peptide derived from the regions between amino acids 10 and 28. The peptide 1–18 EIA detected antibodies in only 59.8% of the 254 specimens. This peptide detected a statistically significant greater proportion of individuals infected with genotype-1 than those infected with...
genotype-3 (77.7% vs. 42.1%, \( P < 0.0001 \), see Table I). This differential was also apparent when comparing all type-1 or type-2-infected individuals with those infected with type-3, 4, or 6 (73% vs. 43%). In addition, the mean signal-to-negative ratio for the 1–18 peptide assay was higher for genotype-1 specimens compared to non-type-1 (47.1 vs. 20.2, data not shown). Examination of consensus HCV core amino acid sequences for each of the six major genotypes (Fig. 2) reveals the existence of mutations at positions 4 and 16 among types 3 and 6 relative to genotype-1 and 2, that is Asn to Leu at position 4 and Asn to Ile at position 16. The mutation of the Asn4 and Asn16 residues to amino acids bearing polar side groups may significantly change the conformation of the epitope(s) within this region thus leading to diminished reactivity to type 1a-derived peptides by non-type-1 or -2-infected individuals.

There was reduced detectability of type-3 over type-1-infected individuals when using peptide 10–24 (Table I, \( P = 0.0002 \)). However, this peptide detected anti-core antibodies in many more type-4 and -6-infected individuals than peptide 1–18. While the Asn-to-Ile mutation at position 16 may influence type-specific reactivity for peptide 1–18, this is not the case for peptide 11–28 which detects 85% of type-1-infected individuals and 89% of those infected with type-3. In addition, peptide 11–28 exhibits reduced reactivity among type-4-infected individuals compared to peptide 10–24 though the Glu-to-Met mutation at position 20, observed in both type-4 and -6 consensus core sequences, is spanned by both peptides. The reason for this apparent discrepancy is unclear though the presence of other non-consensus mutations among type-4-infected individuals may be a contributing factor. Interestingly, peptide 96–120 demonstrated somewhat better reactivity toward genotype-3-infected individuals over those infected with genotype-1 (\( P = 0.039 \); Table I). A trend toward higher reactivity of type-1 over type-2-infected individuals was apparent for peptides 61–78 and 106–130 though this did not reach statistical significance.

### TABLE I. Detection of HCV Core Antibodies by Individual Peptide EIAs. Percent of Anti-HCV-Positive Human Serum Specimens Infected With Genotype-1, 2, 3, 4, or 6 Are Shown

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<td>72.0</td>
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<td>58.9</td>
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<td>94.4</td>
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<td>71.4</td>
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<td>28.6</td>
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<td>33.1</td>
<td>23.6</td>
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Peptide assays showing statistically significant difference in detection rate compared to genotype-1-infected individuals are indicated by “a.”

\( ^{a}P < 0.05 \) versus genotype 1 (Fisher’s exact test).

Influence of Sequence Diversity

Analysis of the HCV core amino acid sequences from a subset of the 254 specimens revealed significant sequence differences between the core protein and some peptides. As shown in Figure 3, there is considerable variability within the core region from amino acids 60–89 among a subset of the specimens tested.
which display a wide range of S/N values in the peptide 61–78 EIA. There are several instances where the core sequence obtained from the donor exhibits seven mutations relative to the peptide sequence though anti-core antibodies are still detected in the assay with S/N values well above the provisional 5.0 cutoff value. In contrast, there is one sample (u34) whose HCV core sequence matches perfectly with that of the peptide sequence and yet is completely unreactive in the assay. 

The ability of the EIA to detect antibodies in some individuals despite such a large number of mutations relative to the peptide sequence may be due to the existence of more than one epitope within the peptide. Indeed, the region from 61 to 67 is conserved in all but one isolate (u24) which is negative in the assay. This region (i.e., 59–68) was shown previously by Sallberg et al. [1994] to contain a major epitope. The high degree of sequence variability within the core region from 61 to 78 of this small sampling of the entire panel may reflect the variability present in the population as a whole but cannot alone explain the overall low reactivity of this peptide (i.e., 51.6% of the 254 samples).

Effectiveness of Longer Peptides

As noted above, and illustrated by the data in Figure 1 and Table I, peptide 10–43 detected antibodies in 100% of specimens. The peptide with the next highest sensitivity was 1–30, which detected antibodies in 92.9% of all individuals. The enhanced sensitivity of these longer peptides compared to assays using 15–18-mers may be due to the ability of longer peptides to adopt a more native conformation thus displaying epitopes more favorably. It is not likely due to simply ligating multiple epitopes together into a single molecule. If this was the case, it should be possible to demonstrate 100% detection by combining results from individuals peptides. Indeed this was not possible, the highest percentage detected by combining results for two peptides was 95.9% (10–24 + 11–28). Even if positive results from all of the individual peptide assays were combined (but excluding peptide 10–43), we could still only detect 251 of the 254 antibody-positive specimens, thus, there were three anti-core-positive individuals non-reactive to any of the short peptides (including peptides 1–30). All three of these were individuals infected with genotype-3a. However, use of peptide 10–43 detected core antibodies in these three individuals. Thus, the core peptides with the best ability to detect antibodies appear to be those that are longer than 30 residues and that do not overlap amino acids 1–10. Omission of sequences that include position 4, which exhibits Arg-to-Leu substitutions in genotypes 1 and 6 (based on consensus sequences), appears to eliminate genotype-dependent anti-core detection.

DISCUSSION

We have analyzed the genotype-dependent reactivity of anti-HCV-positive individuals against a panel of overlapping HCV core peptides. All peptides were derived from the prototype genotype-1a sequence and serum samples were from individuals infected with five of the six major HCV genotypes (no sera from genotype-5-infected individuals were identified). In addition, all individuals possessed core antibodies as determined by using an EIA consisting of a core recombinant antigen encompassing amino acids 1–150. The shortest peptide with the ability to detect the most individuals was peptide 10–24; this 15-mer detected 89.4% of the 254 antibody positives. However, this peptide also displayed a bias for detection of individuals infected with HCV genotype-1 versus genotype-3 (90.8% vs. 79.4%, \( P = 0.0002 \)). Examination of the consensus sequences of the core protein for each genotype (Fig. 2) reveals a substitution of an isoleucine for asparagine at position 16 among genotype-3 sequences. Hence, the genotype-dependent reactivity for this peptide correlates with the presence of this mutation. Similarly, peptide 1–18 shows a bias toward detection of genotype-1-infected individuals over those infected with genotype-3 (\( P = 0.0001; \) Fig. 1, Table I). Consensus sequences reveal the existence of a leucine substitution at position 4 among genotype-3 core sequences compared to genotype-1 sequences. Only two other short peptides displayed statistically significant genotype-1-specific reactivity over one other genotype, that is, peptides 11–28 and 96–120. Interestingly, peptide 96–120 actually detected a higher percentage of genotype-3-infected individuals than genotype-1. The reason for this apparent contradiction is unknown and would require sequencing the core gene from all genotype-1 and -3-infected individuals to determine whether the existence of non-consensus mutations correlated with immunoreactivity. Peptide 1–30 detected antibodies in more samples than the shorter peptides that overlap this region (92.9%; Table I) and peptide 10–43 detected antibodies in 100% of the specimens. Hence, long core peptides that include the more highly conserved region between amino acids 1–40 display little or no genotype-dependent immunoreactivity. In fact, we detected no such dependence for 18-mer peptides made from sequences between amino acids 21 and 78 (Fig. 1 and Table I).

In a previous report, Leon et al. [1998] examined a cohort of 87 EIA 2.0-positive, RIBA anti-core indeterminate blood donor samples for reactivity to multiple HCV core peptides. They indicated that among the donors who were RIBA-3 and anti-E2-negative, most (i.e., 22/31 or 71%) were reactive to a peptide corresponding to amino acids 1–15. As these individuals were also HCV RNA-negative, the authors concluded that the peptide 1–15 reactivity was non-specific. But, since RIBA-3 uses a peptide derived from amino acids 10–53 of core, antibodies against epitopes within the first 10 amino acids of core would not be detected by RIBA-3. In addition, some RIBA-3-negative individuals with peptide 1–15 antibodies also exhibited antibodies against other core peptides. Furthermore, the authors used only genotype-1a-derived peptides. Our peptide corresponding to amino acid 1–18 detected 77.7% of
genotype-1-infected anti-HCV-positive individuals but only 42% of genotype-3-infected individuals ($P = 0.0001$; Table I). Thus, it is possible that the use of a genotype-1 core peptide resulted in an underestimation of the prevalence of antibodies directed against the core 1–15 region. It is also possible that because our peptide was 3 amino acids longer that epitope(s) were presented in a more immunoreactive conformation. It may not be accurate to conclude that immunoreactivity to amino acids 1–10 of core among HCV RIBA-3 indeterminates is due to non-specific cross-reactivity. It is also possible that the apparent selective reactivity to peptide 1–15 is characteristic of resolved infections.

El Awady et al. [2002] demonstrated excellent sensitivity (i.e., 99/100, 99%) of an HCV core peptide-based immunoassay for detection of individuals infected predominantly with genotype-4 despite the use of a peptide (amino acids 21–40) based on a consensus sequence with identity to genotype-1. This is in contrast to the detection rate exhibited by our analogous peptide comprising amino acids 21–37 which detected 22.4% of all samples and only 27.8% of genotype-4-infected individuals (Table I). The differences in detection rates may be due to differences in the length of the peptides used or differences in assay sensitivity.

Utilization of short HCV core peptides (18–24-mers) does not allow for highly efficient detection of known HCV core antibody-positive samples, where anti-core positivity is determined using a recombinant antigen encompassing the majority of the core protein (1–150). The highest detection rate achieved by using peptides 18–24 residues in length was 89.4% (peptide 10–24). Similarly, combining results from assays using short peptides, while increasing detection rates to > 95% (using a combination of peptides 10–24 and 11–28) did not allow detection of all anti-core positives.

Combining results for all peptides used in the study still did not allow for detection of all 254 anti-core positives. However, a longer peptide, 1–30, detected 92.9% of all anti-core-positives and eliminated genotype-dependent reactivity, and peptide 10–43 detected 100% of the specimens. Thus, the high sensitivity of long peptides for anti-core detection may be due to the ability of longer peptides to adopt a more native conformation thus displaying epitopes more favorably. Given that most commercial diagnostic and blood screening assays utilize genotype-1-derived, full-length or near full-length recombinant core antigens for antibody detection, it is not likely that false negative results are due to an inability to detect core antibodies. Though there have been reports of diminished signal intensity upon testing of individuals infected with non-type-1 isolates [Dhaliwal et al., 1996; Neville et al., 1997] no false negatives resulted. As stated by Dhaliwal et al. [1996], commercial antibody assays incorporating genotype-1-derived recombinant antigens may exhibit diminished signal intensity when used to screen populations where non-type-1 infection predominate, however, the fact that these assays incorporate proteins from the NS3 and NS4, and in some cases NS5, the risk of false negatives is diminished. Arguably, the highest risk for false negatives would likely arise during early seroconversion when antibody titers are lowest. Newer combination assays that allow simultaneous detection of core antigen and antibodies [Shah et al., 2003; Laperche et al., 2005] diminish the risk of false negatives during early seroconversion even further. New antibody assay designs should consider use of synthetic peptides of at least 30 residues from the core immunodominant region if not full-length core antigens. If short peptides are preferred, multiple overlapping peptides would be needed to achieve the sensitivity achieved using 30-mer or longer polypeptides.

In summary, it was shown that short peptides derived from the amino terminus of core demonstrate a genotype-dependence in their ability to detect core antibodies among known anti-core-positive donors. While sequence variability plays a significant role in the ability of a peptide assay to detect antibodies among individuals infected with non-type homologous isolates, this dependence is eliminated by using peptides spanning multiple epitopes within the immunodominant region of core.

REFERENCES


