Conformational Epitopes Detected by Cross-Reactive Antibodies to Envelope 2 Glycoprotein of the Hepatitis C Virus

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The prevalence of anti-E2 antibody in persons chronically infected with hepatitis C virus (HCV) is high irrespective of viral genotype, and this cross-reactive antibody is thought to react with a conformational epitope. To investigate the characteristics of this anti-E2 antibody, the immunoreactivity of sera from HCV-1b–infected patients was measured against various modified forms of E2 glycoprotein derived from HCV-H (genotype 1a) by an immunofluorescence technique. Twelve of 18 patients were positive for anti-E2 antibody, and 10 of the 12 required a minimal amino acid (aa) region including aa 406–644 for strong reactivity, suggesting that the major E2 antibody has a conformational epitope in this region. Subsequent analysis using mutant E2 glycoproteins designed to lose N-glycosylation potential at varying sites revealed seven important N-glycosylation sites in this region. Four of these (aa 423, 430, 448, and 576) are indispensable for an antibody response.

A first step in designing a vaccine is the identification of components involved in protective immunity. Little is known about the role of the immune response in the course of hepatitis C virus (HCV) chronic infection. However, because the first contact between a virus and its host cell occurs via binding of the viral envelope to cell-surface receptors, neutralization of this interaction would be anticipated to represent a major mode of preventing infection. Accumulating evidence suggests a potential neutralizing role for antibodies directed at the viral envelopes E1 and E2. Successful protection of chimpanzees was achieved after immunization with recombinant E1 and E2 proteins [1]. In this model, a correlation was established between titers of induced anti-E2 antibodies and protection. These findings suggest that anti-E2 antibody would be important for a successful hepatitis C vaccine.

Previous studies [2–5] have shown that the prevalence of anti-E2 antibody among chronically HCV-infected patients is as high as that of core antibody regardless of viral genotype, even though the E2 sequence shows far less homology among genotypes than the core sequence. A specific cell-binding assay developed by Rosa et al. [6] showed that the ability to neutralize binding of E2 glycoprotein derived from the HCV-1a strain is distributed equally among sera from persons infected by HCV genotypes 1, 2, and 3. These observations raise a possibility that such a cross-reactive anti-E2 antibody, which is prevalent among infected persons, could be protective against HCV infection.

While some reports [2–5] have shown a high prevalence of anti-E2 antibodies in HCV-infected persons, a few surveys [7, 8] found a much lower prevalence. This divergence of results is thought to arise from a difference in the conformation of the E2 protein utilized in the antibody detection system; some researchers [3–5] used glycosylated undenatured E2 protein, but others [7, 8] used nonglycosylated or denatured protein. These observations indicate that the epitope of broadly cross-reactive anti-E2 antibody is not linear but conformational, an impression confirmed by Lee et al. [2]. In the present study, we focused on this conformational antibody. Of two methodologically similar reports [3, 4] concerning antibody responses of patient sera using E2 glycoprotein encompassing amino acids (aa) 388–664 of the HCV-H strain [9] (genotype 1a), one study [3] noted a prevalence of cross-reactive antibodies as high as 69% in HCV-1b–infected patients. Based on this observation, we used an immunofluorescence technique to measure the antibody response of HCV-1b–infected patient sera against modified forms of E2 proteins derived from the HCV-H strain.

Materials and Methods

Patients. We studied 18 randomly selected patients with HCV-1b at the Nagoya University Hospital. All had chronic hepatitis and were studied before any interferon treatment. The patients were seropositive for HCV antibodies by a second-generation assay and had HCV RNA genotype 1b, determined by the second-generation method of Okamoto et al. [10]. Patient sera were negative for hep-
atritis B surface antigen, hepatitis B core antibody, and antinuclear antibodies, and the clinical histories indicated no other causes of hepatitis.

**Plasmid construction.** Plasmids used in this study are summarized in figure 1. The aa positions are numbered in terms of the HCV-H strain. Construction of the different plasmids containing partial E2 sequences, all derived from HCV-H, has been described [11]. A backbone expression plasmid, pCI with a cytomegalovirus promoter followed by an intron, was purchased from Promega (Madison, WI). All plasmids were designed to include the preceding region of the E2 sequence, spanning the residues aa 348–383, which include a putative signal peptide sequence of the E2 protein. Mutations of pCI406–644 were established using a Transformer site-directed mutagenesis kit (Clontech Laboratories, Palo Alto, CA). All plasmids in this study were validated by direct sequencing of the inserted region.

**Immunofluorescence study.** Human malignant melanoma cells (Mewo JCRB0066, purchased from Health Science Research Resources Bank, Osaka, Japan) were cultivated in RPMI 1640 medium containing 10% fetal bovine serum supplemented with gentamicin and penicillin. Transfection of various plasmids to the cells was performed using Lipofectamine (GIBCO BRL, Gaithersburg, MD). Forty-eight hours after transfection, the cells were washed with PBS, fixed with 2% formalin/PBS, and then permeabilized with 1% saponin/PBS. Expressed cytoplasmic E2 protein was detected by an indirect immunofluorescence technique using patient sera (diluted 1 : 50 in PBS) and an anti-human immunoglobulin G±fluorescein isothiocyanate conjugate (Sigma-Aldrich Japan, Tokyo, Japan; 1 : 20 in PBS). Reactivity of patient sera was assessed under fluorescence microscopy and classified into three categories according to fluorescence intensity as strongly positive (+ + +), positive (+), or negative (--). Negative and positive controls were pCISS and pCI384–674, respectively. Reactivity of each serum sample was measured in duplicate for each experiment.

**Results**

**Prevalence of anti-E2 antibody in HCV-1b–infected patients.** An immunofluorescence study using pCI384–674 revealed that 12 of 18 HCV-1b–infected patients were anti-E2 antibody–positive. In all subsequent experiments, sera from the 12 anti-E2–positive patients were investigated (patients 1–12, table 1).

**Epitope mapping of the cross-reactive anti-E2 antibody (experiments 1–5).** Immunofluorescence reactivity of sera to various parts of the E2 protein expressed by plasmid-transfected cells was analyzed (figure 1). For each experiment, new plasmids were designed according to data from previous experiments, and the minimal aa range required for major positive reactivity of sera was determined in a stepwise manner for experiments 1–5. The results of experiments 1–3 revealed that the C-terminus of the major anti-E2 epitope is cysteine at aa 644, and experiments 4 and 5 showed that its N-terminus is glycine at aa 406. Further details of reactivity of individual sera to various parts of the E2 protein are summarized in table 1.

**Contribution of N-glycosylation sites (experiment 6).**

The aa sequence of the HCV-H strain includes 10 triplets forming potential N-glycosylation sites (figure 2: asparagine-X-threonine; X representing any aa) in the major anti-E2 epitope ranging from aa 406 to 644. Comparative study of the HCV-H strain and various HCV-1b strains obtained from GenBank revealed 9 conserved plus 1 nonconserved (at aa 476) N-glycosylation sites in this region (figure 2). To investigate the contribution of these sites to the reactivity of anti-E2 antibody, we constructed 10 additional plasmids (figure 1, experiment 6) by introducing mutations into pCI406–644. These mutant plasmids were designed to substitute glutamic acid for asparagine at 1 of the potential N-glycosylation sites, resulting in lost glycosylation potential without much alteration of protein conformation. As shown in table 1, mutations at aa 423, 430, 448, and 576 resulted in the complete loss of reactivity, while mutations at aa 476, 532, and 540 did not alter reactivity in any positive patient. As the sequences of all the plasmids had been validated, the absence of reactivity of the former mutations, which was confirmed by two additional experiments with positive controls, was thought not to result from experimental error.

**Discussion**

The lack of a sensitive in vitro tissue culture assay for HCV has impeded the testing of the neutralizing potential of antibodies. Nonetheless, progress has been made in the development of substitute assays. Rosa et al. [6] have developed a specific cell-binding assay in which the purified glycoprotein E2 (aa 384–715) is quantitatively adsorbed onto susceptible cells. Antibodies capable of neutralizing the binding of E2 in this assay (neutralization-of-binding [NOB] antibodies) have been detected in up to 40% of chronically infected persons. In addition, protection of chimpanzees immunized with envelope glycoprotein was correlated not only with high anti-E2 antibody titers but also with high titers of NOB antibodies. Using this binding assay, they observed at least two neutralizing epitopes on the E2 glycoprotein: one is in hypervariable region 1 (HVR-1), and the other is thought to reside in the region relatively conserved among several genotypes. Because a broadly protective vaccine requires the induction of a cross-reactive antibody to a conserved epitope, the latter epitope represents a candidate vaccine component.

The prevalence of cross-reactive anti-E2 antibody in HCV-1b–infected patients in this study (12 of 18 patients) is similar to previous data (69%, reported by Yuki et al.) [3]. In our subsequent experiments (experiments 1–5), the antibody response of all anti-E2–positive sera was reduced by the absence of the N-terminal glycine at aa 406 or the C-terminal cysteine at aa 644 (absence of alanine at aa 643 in patients 2 and 9). This result pinpoints the major anti-E2 epitope to the aa 406–644 segment (aa 406–643 in patients 2 and 9). Strong reactivity always requires the simultaneous presence of both terminal residues of this segment, suggesting that an identical an-
Figure 1. Schematic diagram of expression plasmids. All constructs were based on pCIE2SS, which includes putative signal peptide sequence of E2 protein (E2SS). Partial or mutant E2 sequences derived from strain hepatitis C virus (HCV)-H were inserted into position subsequent to E2SS in pCIE2SS. Constructed plasmids were named by pCI amino acid (aa) position numbers that represent range of inserted E2 segment. Experiments 1–3, 4, and 5 were designed to determine C- and N-terminus of epitope of cross-reactive anti-E2 antibodies, respectively. Experiment 6 investigated involvement of N-glycosylation as described in text. Ten mutant plasmids based on pCI406–644 were constructed. They were designed to substitute glutamic acid for asparagine at potential N-glycosylation sites in figure 2, resulting in loss of N-glycosylation potential at each site. Mutant plasmids were named by pCI406–644m position no. of mutated aa. Reactivity most prevalent among 12 anti-E2-positive sera (major reactivity) is shown at right of each plasmid.
Table 1. Reactivity of sera from anti-E2-positive patients to various modified forms of E2 protein expressed by plasmid-transfected human melanoma cells.

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<th>Patient</th>
<th>pCI</th>
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The antibody recognized the two terminal aa, 406 and 644 (643 in patients 2 and 9). As the two termini are not close to each other one-dimensionally, the epitope of this antibody appears to be not linear but conformational, and the two termini are close in the three-dimensional protein configuration. Comparative study of various HCV-1b strains revealed that the terminal aa of these epitopes at aa 406, 643, and 644 are almost conserved (figure 2). In contrast, the aa at positions 407, 408, and 641, adjacent to the conserved termini, showed high variability (figure 2). Because variable aa regions are unlikely to be involved in an epitope recognized by a cross-reactive antibody, these variable positions located near both termini are unlikely to serve as a direct epitope, although they may contribute to epitope conformation.

Results in half of the seropositive patients (patients 1, 3, 4, 6, 8, and 10) cannot be explained only by the antibody described above. Patients 3, 6, and 8 were characterized by reactivity of pCI466-644 but no reactivity of pCI384-643. This observation supports the existence of another epitope in the range of aa 466-644, where the C-terminal cysteine at aa 644 is essential. Patients 4 and 10 showed reactivity of pCI384-554 but no reactivity of pCI407-644. This supports the existence of an additional epitope involving aa 384-406; HVR-1 may be one candidate for such an epitope. Thus, some epitopes other than the major epitope (aa 406-646) are important, but they seem to be less prevalent among HCV-infected patients and apparently induce less intense reactivity.

No difference was apparent in reactivity between pCI384-644 and pCI406-644 among all positive patients, suggesting that a major part of HVR-1 (aa 384-405) cannot contribute to induction of a sufficiently intense cross-reactive antibody. The finding that pCI384-554, containing the entire HVR-1 sequence, does not have widespread reactivity supports this hypothesis (only 3 of 18 patients were positive). In interpreting our results, however, one must consider that the immunofluorescence method used in this study might be less sensitive than other detection systems (e.g., ELISA or immunoblotting). Because we limited our focus to a broadly cross-reactive antibody with strong reactivity, the methods we chose for this study are likely to be sufficient. Nonetheless, many authors [12-15] have reported the existence of linear epitopes in HVR-1 by ELISA. Some of these studies demonstrated that an HVR-1 antibody had an important neutralization site [12, 13] and showed cross-reactivity regardless of genotype [14, 15]. However, Hattori et al. [14] have suggested that HVR-1 antibodies do not efficiently neutralize HCV, while antibodies to conformational epitopes may be more important for neutralization.

Among the 10 mutations at the potential N-glycosylation sites, only 3, at aa 476, 532, and 540, did not affect reactivity. The other 7 mutations apparently reduced the reactivity of the sera, indicating their involvement in determining the conformational epitope. In particular, the mutations at aa 423, 430, 448, and 576 resulted in a complete loss of reactivity, suggesting that N-glycosylation at these sites is indispensable to the induction of this antibody. Of interest, the degrees of influence upon reactivity vary widely between the 10 mutations. Judging from the difference in requirement of N-glycosylation sites, the cross-reactive anti-E2 antibody may not be homogeneous, con-
Figure 2. Amino acid (aa) sequence (406-644) alignment of hepatitis C virus (HCV)-H (genotype 1a) and HCV genotype 1b strains deduced from GenBank (indicated by accession no.). aa residues are indicated by standard single-letter codes; dashes indicate residues identical to those in HCV-H strain.

...sisting of multiple antibodies with different epitopes whose N-terminus is fixed at aa 406 and whose C-terminus may be either aa 643 or 644.

In this study, we demonstrated the characteristics of putatively conformational epitopes of cross-reactive antibodies in HCV E2. Although we did not establish that these antibodies could prevent HCV infection, we strongly believe that our data are valuable for HCV vaccine construction or helpful for determining the three-dimensional structure of native E2 protein on the virus surface.

References


