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Epitope Mapping of Antibodies Directed against Hypervariable Region 1 in Acute Self-Limiting and Chronic Infections due to Hepatitis C Virus

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Epitopes of hypervariable region 1 (HVR1) were mapped by enzyme-linked immunosorbent assay using follow-up sera of patients, all of whom were infected with the same isolate of hepatitis C virus (HCV). Our results suggest that (i) an early appearance (up to month 13 postinfection) of antibodies directed to the N terminus of HVR1 is associated with acute self-limiting infections of HCV and (ii) isolate-independent antibodies which are mainly directed to the C terminus of HVR1 seem to persist in chronically infected patients. The relevance of HVR1-specific antibodies for neutralization was evaluated by characterization of a rabbit serum.

Hepatitis C virus (HCV) has been acknowledged as a major etiologic agent of acute and chronic hepatitis around the world since the cloning of its cDNA in 1989 (3). Chronic infections develop in 50 to 80% of patients and are often followed by cirrhosis and more rarely by hepatocellular carcinoma (1). The molecular mechanism involved in the high rate of viral persistence is not understood. Reinfection by HCV has been observed in chimpanzees (9) and may also occur in patients (8, 21, 22). On the other hand, protection from HCV infection could be achieved by vaccination of chimpanzees with the putative HCV envelope proteins E1-E2 (2). However, this protection seemed to be dependent on the viral isolate used for challenge. It has been suggested that one mechanism involved in the establishment of viral persistence is the immune escape of HCV variants (23) which have mutations in B- and T-cell epitopes (28, 30). Amino acid changes observed frequently in a region of about 27 amino acids, termed hypervariable region 1 (HVR1), which is located at the N terminus of envelope protein E2 (13, 16, 17, 29) were postulated to lead to a viral escape from neutralizing antibodies (30). Different approaches were used to show that neutralizing antibodies exist. In the chimpanzee model of HCV, antibodies present in patient sera could prevent infection by HCV when incubated in vitro with virus prior to infection (10). Recently, an HVR1-specific hyperimmune serum was also shown to partly prevent infection by HCV (11). Studies of tissue culture cells which support replication of HCV also suggested the existence of neutralizing antibodies. Antibodies which could prevent binding of virus (26) or recombinant E2 protein (24) to such cells were implicated in the neutralization of HCV. From these findings, it was inferred that HVR1-specific antibodies neutralized virus (24, 33). HVR1 contains various B-cell epitopes (15, 18, 27, 30), but it is not known whether the recognition of certain epitopes during the first few months of infection correlates with the elimination of virus in patients.

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We addressed this issue by epitope mapping of HVR1 (main variant) of isolate HCV-AD78 by using sera of patients who were all infected by this isolate, which was present in an HCV-contaminated anti-D immunoglobulin (6). Patients were monitored for up to 17 years postinfection (p.i.). One group of patients was diagnosed retrospectively as having acute self-limiting HCV infection by the positivity of the patients' anti-HCV reactivities (second- and third-generation enzyme-linked immunosorbent assay [ELISA] [Abbott, Chicago, Ill.]; immunoblot assay [Ortho Diagnostics, Raritan, N.J.]), by the absence of HCV RNA in follow-up examinations, and by initially elevated alanine aminotransferase (ALT) levels that dropped thereafter. A second group of patients that developed chronic HCV infections was characterized by positivity of anti-HCV reactivity and the presence of HCV RNA throughout follow-up, by maintenance of elevated ALT levels, and by histological features of chronic persistent hepatitis after liver biopsies. Patient sera which have been selected by the presence of antibodies directed to peptide A1 (see below) and by time point of infection were used for epitope mapping.

Epitope mapping of HVR1. The amino acid sequence of the main variant of HVR1 of HCV-AD78 was determined by sequence analysis (14, 33). The complete sequence of the main variant as well as those of three minor variants (about >50, 23, 15, and 8% of cDNA clones) were represented by peptides termed A1, B1, C1, and D1, respectively (Fig. 1). The sequence of peptide A1 was subdivided for epitope mapping into 20 decamers, termed A01 to A20, that overlap by nine amino acids. Decamers were grouped (group N and group C) according to their location within HVR1 (Fig. 1). An ELISA was performed essentially as described previously (32) by using HVR1-specific peptides which each carry a biotinylated spacer peptide at their N termini (19). Patient sera were diluted 1:250 and were scored positive by ELISA when values of optical density at 405 nm (OD_{405}) were above 0.2 (cutoff, three times mean negatives + 10%).

Epitope mapping of sera obtained early p.i. Sera of 12 patients who had acute self-limiting infections of hepatitis C (HCV-AD78) and sera of 11 patients who had chronic infections were investigated first (Fig. 2A and B, respectively). Sera were obtained early after infection (up to month 13 p.i.). Pep-

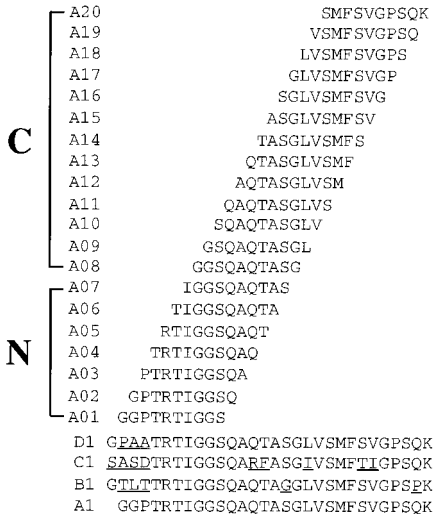

FIG. 1. Amino acid sequences of peptides. Peptide A1 (29 amino acids) represents the complete main variant of HVR1 of HCV-AD78, while peptides B1, C1, and D1 represent minor variants. Peptides A01 to A20 are overlapping decamers covering the sequence of the main variant (peptide A1). Amino acids which differ with respect to those of peptide A1 are underlined. N and C refer to groups of decamers (see text).

tides A1, B1, C1, and D1 were recognized by all patient sera, although the relative OD_{405} values for individual peptides varied. The four peptides representing the complete HVR1 were recognized with the same frequency in the two patient groups. Also, most patient sera recognized more than one decamer. Only serum of patient a4 (Fig. 2A) did not react with any of the decamers, suggesting the existence of epitopes which cannot be represented by 10 amino acids. Decamer A20 was the most frequently recognized individual decamer (sera from 8 of 12 patients in the group with self-limiting HCV infection and from 9 of 11 patients in the group with chronic HCV infection [Fig. 2A and B, respectively]). Other decamers, which include the highly divergent N termini of the other three variants of HVR1 (Fig. 1), were not recognized by these sera (data not shown), suggesting that the main variant represented by peptide A1 is immunodominant after infection by HCV-AD78.

The pattern of individual decamers recognized by antibodies was unique for each patient, suggesting a patient-specific induction of antibodies against the main variant of HVR1 (Fig. 2). The epitopes mapped throughout the entire sequence of HVR1. However, epitopes could be grouped according to their location within HVR1 (Fig. 1). The decamers of group C were recognized more frequently (21 of 23 sera) than the decamers of group N (9 of 23 sera). All sera of patients (11 of 11) who developed chronic infections contained antibodies directed to decamers of group C (Fig. 2B); 10 of 12 sera of patients who had acute self-limiting infections also contained such antibodies (Fig. 2A). In eight of nine sera which contained antibodies against group N decamers, additional antibodies which recognized decamers of group C were present. The only exception was the serum of patient a6 (Fig. 2A).

With regard to the two courses of infection, the presence of antibodies directed to group N decamers differed between patient groups. In 8 of 12 patients who had an acute self-limiting infection such antibodies were detected (Fig. 2A). In contrast, only in 1 of 11 sera (patient c9) of patients who developed chronic infection could these antibodies be observed (Fig. 2B). This difference between patient groups was

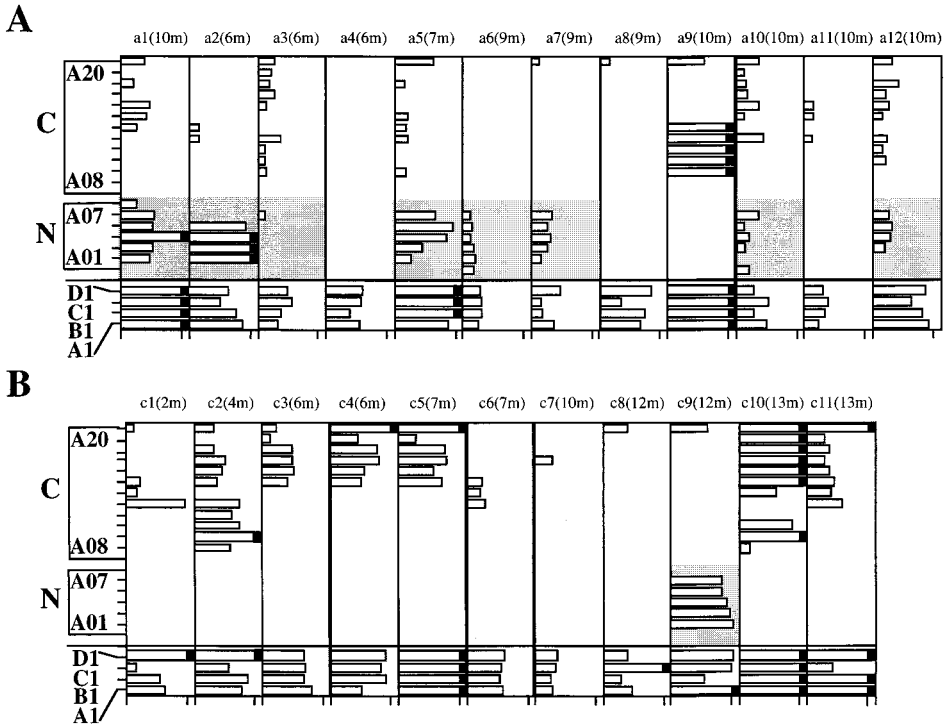

FIG. 2. Mapping of antibodies in patient sera with peptides A1 to D1 or decamers A01 to A20. Sera were obtained from patients having acute self-limiting HCV infection (patients a1 to a12) (A) or chronic HCV infections (patients c1 to c11) (B). OD_{405} values are plotted on the *x* axis. Only OD_{405} values between 0.2 (cutoff) and 0.6 are given. OD_{405} values above 0.6 are indicated by black bars. Patient sera were diluted 1:250. The month of serum drawing p.i. is indicated in parentheses after the patient designation. N and C refer to the groups of decamers shown in Fig. 1. Shading is used to indicate that one or more decamers of group N were recognized.

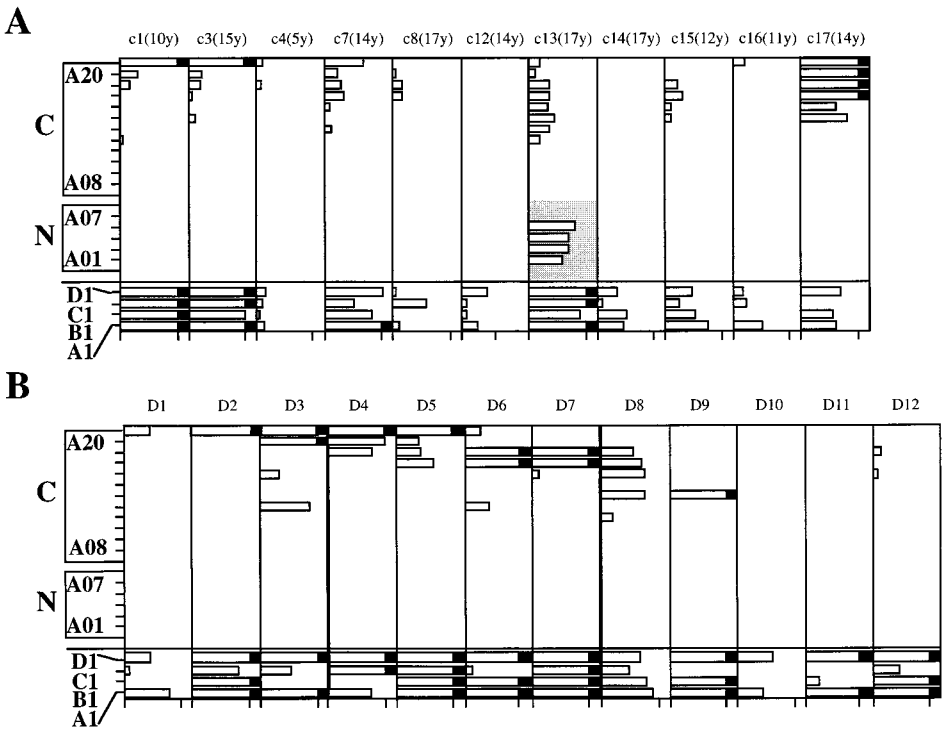

FIG. 3. Mapping of antibodies from sera of chronically infected patients with peptides A1 to D1 or decamers A01 to A20. Sera were obtained from patients with chronic infection by HCV-AD78 (A) and from other chronically infected patients (B). OD_{405} values are plotted on the *x* axis. Only OD_{405} values between 0.2 (cutoff) and 0.8 are given. OD_{405} values above 0.8 are indicated by black bars. N and C are as defined in the legend for Fig. 2. The year of serum drawing p.i. is indicated in parentheses after the patient designation.

found to be significant ($P < 0.005$ as determined by the χ^2 [two-tailed] method).

Epitope mapping of sera obtained from patients with chronic HCV infection. Antibodies to HVR1 were previously observed with high frequency (67%) in the group of patients chronically infected with HCV-AD78 and also, albeit with a lower frequency (15%), in a second group of chronically HCV-infected patients (33). We tested sera from these patient groups (Fig. 3). HVR1-specific antibodies from sera of 11 patients chronically infected during an HCV-AD78 outbreak were obtained between 5 and 17 years p.i. and were mapped (Fig. 3A). HVR1-specific antibodies from sera of 12 chronic HCV patients not affected by this outbreak (Fig. 3B) were also obtained and mapped. Sera of five chronically infected patients (patients c1, c3, c4, c7, and c8) have also been analyzed in the early phase of infection (Fig. 2B). The antibody response against peptides containing the complete sequence of HVR1 (peptides A1, B1, C1, and D1) was not found to be significantly different between the two patient groups. However, compared to that of the early phase of infection (Fig. 2), the pattern of reactivity was less complex. Ten sera did not contain antibodies which recognized peptide B1 and/or C1 (Fig. 3). Four sera did not react with any of the decamers (patients c12, c14, D10, and D11). Decamers A18 and A20 were recognized most frequently (in 14 and 13 of 23 sera, respectively). Decamers of group C were recognized in most of the sera from the two groups of patients (19 of 23 patients and 10 of 12 patients [Fig. 3A and B, respectively]). Decamers of group N were recognized only in one serum (patient c13).

Characterization of rabbit antibodies directed to different epitopes of HVR1 by an HCV in vitro binding assay. A binding assay of HCV to human fibroblasts (33) which were thought to sustain replication of HCV (31) was used to further characterize antibodies directed to different epitopes of HVR1. A rabbit serum (αHVR1A) was chosen for a study of the interference with binding of HCV to these cells in order to exclude the influence of other antibodies present in patient sera which are directed to epitopes outside of HVR1 (24, 33). Serum αHVR1A was generated via immunization of rabbits with purified fusion protein HVR1.A (33) which contains an amino acid sequence identical to that of peptide A1. The rabbit hyperimmune serum recognized peptide A1 as well as peptides B1, C1, and D1 (Fig. 4) and also reacted with decamers of group N (peptides A02 to A07) and group C (peptides A13 to A18) (Fig. 4). The respective preimmune serum did not bind to peptides. Reactive decamers (A02 to A07 and A13 to A18) partially competed (about 50%) the binding of αHVR1A to peptide A1, suggesting that the two groups of antibodies are equally important for binding (data not shown). Inhibition studies of HCV binding by αHVR1A (Table 1) was carried out with 5 μl of HCV-AD78 (about 2.0×10^5 HCV reverse transcription-PCR genome equivalents/ml). Preincubation of HCV-AD78 with low dilutions (1:2 and 1:10) of αHVR1A prevented binding of virus to fibroblasts (Table 1). In contrast, preincubation of αHVR1A with excess amounts of different peptides revealed that peptide A1 and a mixture of decamers A02 to A07 restored binding of HCV, whereas a mixture of decamers A13 to A18 could not (Table 1), suggesting that antibodies directed to the N terminus are sufficient for the observed interference.

Our findings presented above suggest that the antibody response directed to HVR1 can be distinguished according to the course of infection to be directed either against the N and C termini or against the C terminus alone. In patients developing

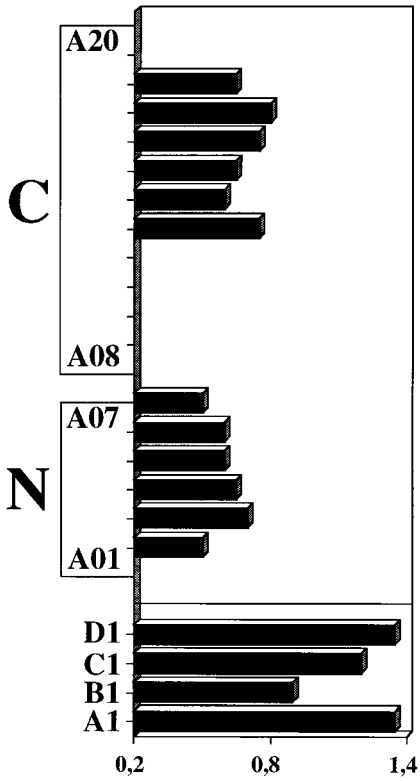

FIG. 4. Mapping of antibodies present in rabbit antiserum αHVR1A with peptides A1 to D1 and decamers A01 to A20. Rabbit serum was diluted 1:2,000. N and C are as defined in the legend for Fig. 2.

or having chronic infections of HCV, the antibody response seems to be mainly directed against the C terminus alone (Fig. 2B and 3). At least some of these antibodies seem to be independent of the HCV isolate (Fig. 3B). Isolate-independent antibodies which were present during chronic infection by HCV have been observed before (25). These HVR1-specific antibodies seem to persist, although the sequence of HVR1 changes rapidly (12, 14, 15, 20). The persistence of these antibodies may be due to a stimulation of B cells by the more conserved C terminus of HVR1 (7, 16, 27) via a mechanism described as the "original antigenic sin" (5). The frequency of antibodies against a single sequence of HVR1 (main variant of HCV-AD78) was higher in sera of patients chronically infected by HCV-AD78 than in patients chronically infected by other isolates (32, 33). This suggests that in addition to isolate-independent antibodies (Fig. 3B), isolate-dependent antibodies which are also directed to the C terminus of HVR1 might exist (Fig. 3A).

TABLE 1. In vitro neutralization by HVR1-specific rabbit serum αHVR1A[a]

Dilution of rabbit serum	Peptide(s)[b]	Neutralization[c]
1:2	No peptide	+
1:10	No peptide	+
1:20	No peptide	+/−
1:100	No peptide	−
1:5[d]	No peptide	−
1:5	A1	−
	A02–A07	−
	A13–A18	+

[a] The inoculum for the binding assay was HCV-AD78 in each case.
[b] Peptides (10 μg each) were incubated with rabbit serum prior neutralization assay.
[c] Result of at least three neutralization assays. +/−, mixed results.
[d] Preserum.

Antibodies which recognize the N terminus of HVR1 were observed with a higher frequency (Fig. 2A) than in previous studies which, however, analyzed at most a few chronically infected patients (15, 18, 27, 30). These antibodies were predominantly found in patients who had acute self-limiting infections of HCV (Fig. 2A). Resolving from disease seems therefore to be associated with the early induction of such antibodies, at least in the patient group studied here. Whether resolving from disease is due to neutralization by these antibodies is not known. However, our characterization of rabbit antibodies (Table 1) suggests that the N terminus of HVR1 might be important for the induction of neutralizing antibodies. In addition, the observation of HCV reinfection in patients (8, 21, 22) suggests that antibodies directed to the C terminus alone might not be sufficient for clearance of virus, because reinfected patients are likely to contain these antibodies, as observed in the present study (Fig. 3B) and by others (25).

In addition to neutralizing antibodies which might be directed against HVR1 (11), other B- (24) and T-cell (4) epitopes may also be important for elimination of virus during natural infection or for protection after vaccination (2). HVR1 could, nevertheless, be of interest for the future development of an HCV subunit vaccine, although the preparation of a complex library of HVR1 peptides which can induce a broad neutralizing antibody response is a challenging approach. Further investigation of the significance of anti-HVR1 for elimination of HCV is needed.

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