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Identification of an immunodominant epitope within the capsid protein of hepatitis C virus

(glutathione *S*-transferase/fusion proteins/epitope mapping/immunoblot/ELISA)

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ABSTRACT **We have isolated cDNA clones from the 5′ end of the Hutchinson strain of hepatitis C virus. Sequences encoding various segments of the HCV structural region were fused to the gene for glutathione *S*-transferase and analyzed for the expression of hepatitis C virus-capsid fusion proteins. With a set of these fusion proteins, both human and chimpanzee immune responses to capsid were studied. An immunodominant epitope was located within the amino-terminal portion of capsid that is preferentially recognized by antibodies in both human and chimpanzee hepatitis C virus-positive sera. In addition, analyses of sequential serum samples taken from humans and chimpanzees with either chronic or apparently self-limited infections revealed that a strong anti-capsid response develops rapidly after onset of infection.**

Recently, a portion of the hepatitis C virus (HCV) genome was cloned from the plasma of an experimentally infected chimpanzee (1). DNA sequence homology studies revealed that HCV is distantly related to both the Flaviviridae and Togaviridae families (2, 3). HCV contains a positive-stranded RNA molecule that encodes a single polyprotein ≈2900 amino acid residues in length. This polyprotein is processed into functionally different structural and nonstructural proteins. Based on homology with flavivirus and pestivirus sequences the 5′ end of the genome is thought to encode the major structural proteins, whereas the 3′ end encodes five or more nonstructural proteins. The nucleotide sequences of the structural region from two Japanese HCV isolates and one American isolate (Eur. Patent Appl. 90302866.0) have been determined (3–5). A highly conserved sequence that begins at the first amino acid of the HCV open reading frame and extends approximately to amino acid 190 has been identified as the putative coding region for capsid (3, 4). Lack of an *in vitro* system for propagating HCV has prevented the physical isolation of distinct structural and nonstructural proteins and the subsequent identification of their functional role in the viral life cycle and disease.

An assay for circulating antibodies to HCV has been developed using a superoxide dismutase–HCV fusion protein produced in yeast (6). The superoxide dismutase–HCV polypeptide (C100-3) is composed of 363 amino acids from the nonstructural region of HCV, referred to as NS3–NS4. Results obtained from this assay suggest that HCV is the major cause of both transfusion-associated and community-acquired non-A, non-B hepatitis (6–8).

The C100-3 antigen-based immunoassay has been reported to preferentially detect antibodies in serum from chronically infected patients (6–9). C100-3 seroconversion generally occurs from 4 to 6 mo after the onset of hepatitis and in some cases fails to detect circulating antibodies in patients diagnosed with non-A, non-B hepatitis. In addition, false-positive results have been reported when C100-3 was used to detect antibodies in patients with autoimmune chronic active hepatitis or liver disease caused by a variety of conditions other than HCV (10, 11).

In this study, we describe the expression of several fusion proteins that encode various segments of the capsid region from the Hutchinson strain of HCV. Each of the fusion proteins was screened for serologic reactivity. Sequential serum samples from both acute and chronic HCV-positive donors were tested for the antibody reactivity by immunoblot assay or ELISA. The results demonstrate that most dominant immunoreactive epitopes are located within the amino-terminal portion of capsid and that antibodies directed against these epitopes appear early after infection. This information should be useful for the development of more sensitive and specific tests for both blood screening and clinical diagnosis of HCV.

MATERIALS AND METHODS

Cloning, Subcloning, and Sequence Analysis. The cloning and sequencing of cDNAs coding for structural proteins from the Hutchinson strain of HCV will be reported in detail elsewhere. Briefly, HCV sequences were obtained after PCR amplification of RNA templates from chimpanzee-infected serum by using primers derived from published HCV sequences (3). Three target sequences derived from the putative capsid region were amplified after two rounds of PCR with the following sets of primers: (*i*) 690–694; (*ii*) 693–691; and (*iii*) 14–16 followed by 15–17. Map positions of the various primers used relative to the AUG start codon are as follows: 690, 1–222; 694, 207–223; 693, 144–162; 691, 340–359; 14, 355–373; 15, 359–376; 16, 961–980; and 17, 957–976. To determine the nucleotide sequence, each PCR fragment was isolated and inserted into pUC18 or pBluescript (Stratagene) vectors by standard cloning procedures (12).

The sequence of Sj26, the gene encoding glutathione *S*-transferase (GST), and the various derivatives of this sequence used to create gene-fusion vectors have been described (13). Subcloning of HCV fragments into plasmids pGEX-3X and pGEX-2T (Pharmacia) was done by using conventional cloning procedures (12). DNA sequence was analyzed on a Pharmacia Automated Laser Fluorescence (ALF) sequenator or by standard techniques (14). Oligonucleotides were synthesized on a Pharmacia Gene Assembler and purified by gel electrophoresis before use.

Expression of GST–HCV Proteins. Plasmids containing GST or GST–HCV sequences were transformed into *Escherichia coli* strain W3110. Cells were grown overnight at 37°C in L broth containing ampicillin (50 μg/ml).

For induction of protein expression, cells were diluted 1:150 and grown to an OD_{550} of 0.5 unit. After addition of

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Abbreviations: HCV, hepatitis C virus; GST, glutathione *S*-transferase.

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isopropyl β-D-thiogalactoside (1 mM), cells were incubated at 37°C for an additional 2 hr and harvested by centrifugation. Protein extracts were prepared by boiling bacterial cell pellets in SDS sample buffer. Extracts were analyzed by SDS/PAGE (15).

Immunoblot Analysis. Protein extracts resolved by SDS/PAGE gels were electroblotted onto nitrocellulose filters. Filters were blocked with WB buffer (1% bovine serum albumin/10 mM phosphate, pH 7.5/0.5 mM NaCl/0.5% Tween 40) for 3 hr before addition of serum (diluted 1:500 in WB buffer) overnight. After three washes in WB buffer, filters were incubated with 2 μCi (1 Ci = 37 GBq) of ^{125}I-labeled protein A (NEN) in WB buffer for 1 hr at room temperature. The filters were then washed three additional times, dried, and subjected to autoradiography.

Sera. Chimpanzee serum was obtained from 3- to 7-year-old animals infected with (*i*) the Hutchinson non-A, non-B inoculum (16), (*ii*) chimpanzee plasma taken during the acute phase of infection resulting from this inoculum, (*iii*) various inocula derived from different human posttransfusion or hemodialysis unit infections, or (*iv*) pooled human sera. At least 3 mo before inoculation and during the first year after inoculation, animals were subjected to weekly bleeding and twice-monthly liver biopsies for EM analysis. Blood samples were obtained at 2-week intervals in the second year and monthly intervals thereafter.

Human serum was obtained from a prospective posttransfusion follow-up study (20). The sera has been stored at −70°C since 1969–1972.

All sera were tested for alanine aminotransferase, hepatitis B surface antigen and antibody, and for antibody to hepatitis B core antigen by using RIA or ELISA kits obtained from Abbott. All sera were tested for anti-HCV (C100-3 antigen) by an ELISA assay manufactured by Ortho Diagnostics.

ELISA Assays. The peptide encoded by pCAP-A was diluted to 1 μg/ml in coating buffer (sodium carbonate, pH 9.6) containing 4 M urea. One hundred microliters of this solution (0.1 μg) was added to each well of a Nunc Immulon-96 plate, and protein was absorbed overnight at room temperature. After three washes with phosphate-buffered saline (PBS)/0.05% Tween 20 (PBST solution), wells were blocked for 2 hr at 37°C with 150 μl of dilution buffer (PBST solution/10% goat serum/3% bovine serum albumin). Plates were washed three additional times with PBST solution before addition of 100 μl of sera (diluted 1:50 in dilution buffer). Serum was incubated for 15 min at 37°C, the plates were washed five times with PBST solution, and 100 μl of horseradish peroxidase-conjugated anti-human IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD; diluted 1:2000) was added to the wells for 15 min at 37°C. The plates were then washed five times with PBST solution and incubated with 100 μl of *o*-phenylenediamine solution for 20 min at room temperature in the dark. The reaction was stopped with 50 μl of 3 M sulfuric acid and read using a microtiter plate spectrophotometer equipped with a 405-nm filter.

RESULTS

Construction of GST–Capsid Fusion Plasmids. Three GST–capsid fusions were constructed by inserting contiguous segments of the capsid gene into the GST fusion vector pGEX-3X (Fig. 1). Plasmid pCAP-A contains the first 222 base pairs (bp) of the capsid gene fused in frame with the 3′ end of Sj26. The resulting vector encodes a fusion protein that is comprised of an amino-terminal polypeptide region corresponding to residues 1–221 of GST, an intermediate polypeptide region corresponding to residues 222–227 and defining a cleavage site for protease factor Xa, a smaller linker segment (amino acids 228–234), and a carboxyl-terminal

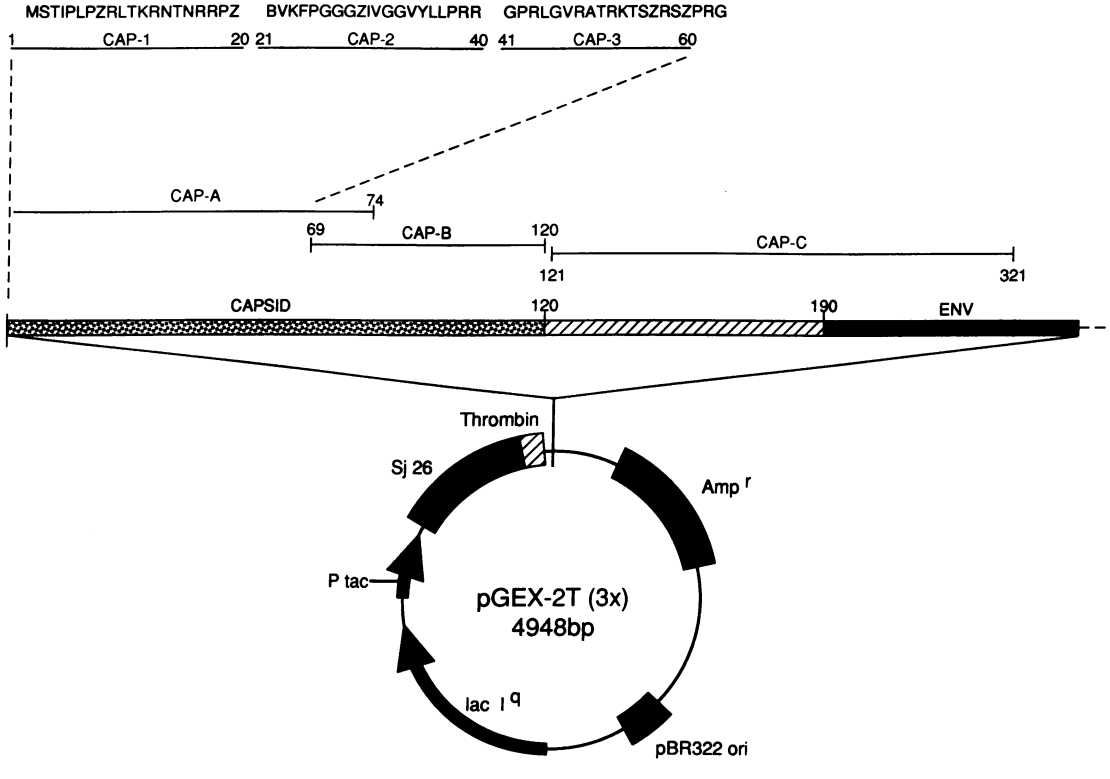

FIG. 1. Schematic representation of the plasmids used. HCV sequences from the region encoding capsid and part of envelope were inserted into the polylinker of pGEX-2T or pGEX-3X downstream of Sj26. Stippled bar represents the region first thought to encode capsid (3). Hatched bar represents additional sequences now thought to encode the carboxyl terminus of capsid (4), and shaded bar represents sequences coding for envelope. Sequences (in one-letter code) of the three fusion proteins identified by CAP-A, CAP-B, and CAP-C and the three subclones of CAP-A-encoding CAP-1, CAP-2, and CAP-3 are depicted above the bar line.

portion corresponding to residues 235–308 and defining amino acids 1–74 of capsid.

Plasmid pCAP-B contains capsid gene sequences 207–360 fused in frame to the 3′ end of Sj26. The resulting fusion protein is comprised of 221 amino acids encoding GST followed by a 6-amino acid recognition site for the protease factor Xa (amino acids 222–227), a 3-amino acid linker segment (amino acids 228–230), and a carboxyl-terminal segment corresponding to residues 231–282 and defining amino acid residues 69–120 of capsid.

Plasmid pCAP-C contains capsid gene sequences 361–570 and *Env* gene sequences (gp33) 571–963 fused in frame to the 3′ end of Sj26. This vector encodes a fusion protein that is comprised of GST at the amino terminus (amino acids 1–221) followed by a 6-amino acid segment defining the factor Xa site (amino acids 222–227), an 8-amino acid linker for the carboxyl terminus of capsid 236–305 (amino acid residues 121–190 of capsid), and the amino-terminal segment of Env (amino acid residues 306–537).

Although the carboxyl terminus of capsid has not yet been identified, amino acid sequence analysis predicts that the sequence encoding capsid ends before nucleotide 570 (4). Thus, taken together, these plasmids contain sequences that span the entire capsid-coding region and partially extend into the envelope-coding region.

To further delineate the location of the seroreactive epitopes within the capsid protein, three smaller GST–capsid fusions were constructed by inserting sets of synthetic oligonucleotides encoding 20 amino acid polypeptides into the GST fusion vector pGEX-2T (see Fig. 1). Plasmid pCAP-1 contains the first 60 bp (amino acids 1–20) of capsid fused in frame to the 3′ end of Sj26. Plasmids pCAP-2 and pCAP-3 contain capsid sequences 61–120 (amino acids 21–40) and 121–180 (amino acids 41–60), respectively, fused in frame to the 3′ end of Sj26. Together, these three fusion proteins contain contiguous capsid sequences that subdivide pCAP-A into three nonoverlapping segments.

Expression of the GST–Capsid Fusion Proteins. Expression of the various GST–capsid fusion proteins was done in *E. coli* strain W3110, as described above. Total cell lysates from induced cells containing plasmids pCAP-A, pCAP-B, PGEX-2T, pCAP-1, pCAP-2, or pCAP-3 were analyzed on SDS/polyacrylamide gels, and proteins were visualized by staining with Coomassie blue, as shown in Fig. 2. Plasmid pGEX-2T encodes authentic GST, which has an apparent molecular mass of 27.5 kDa (lane 2). Plasmids pCAP-A (lane 3) and pCAP-B (lane 4) encode fusion proteins of 36 and 33 kDa, respectively. Plasmid pCAP-C encodes a fusion protein with an apparent molecular mass of 47 kDa (dats not shown). Plasmids pCAP-1, -2, -3 all encode fusion proteins with apparent molecular masses of 29–30 kDa (lanes 5–7). Expression of these capsid fusion proteins varied from 17% of total cell protein for CAP-B to 31% of total cell protein for CAP-3. All fusion proteins, except for CAP-2, were found to be in an insoluble form when expressed in *E. coli*. Minor fusion proteins, which are smaller in size than predicted, probably represent degradation products (Fig. 3, lane 3).

Immunoblot Analysis of Fusion Protein Seroreactivity. Initially, the GST–capsid fusion proteins encoded by plasmids pCAP-A, pCAP-B, and pCAP-C were evaluated for their cross-reactivity with various HCV-positive human and chimpanzee sera. This analysis was done by using immunoblot strips that contained protein extracts similar to the pattern shown in Fig. 2. The results of a typical immunoblot are depicted in Fig. 3. This particular blot was done with NYU201-6, a human serum collected 10 weeks after transfusion from a patient who developed non-A, non-B hepatitis. This serum reacted strongly with CAP-A (lane 3), CAP-1 (lane 5), and CAP-2 (lane 6) and failed to react with CAP-B, (lane 4), CAP-3 (lane 7), and the negative control containing GST (lane 2). Most of the human and chimpanzee sera screened thus far did not cross-react with GST. However, more than half of the HCV-positive sera tested contain one or more antibodies that cross-react with proteins derived from *E. coli*. Thus, GST-fusion proteins that are produced in *E. coli* must be purified to homogeneity to be good candidates for diagnostic test development.

Immunoblot data obtained by using HCV-positive human and chimpanzee sera are summarized in Table 1. The responses to eight chimpanzee (five chronic and three acute) and five human (acute) HCV-positive sera are listed. All of these HCV-positive sera reacted with CAP-A, and none, except 201-12 (weak CAP-B reactor), react with CAP-B or

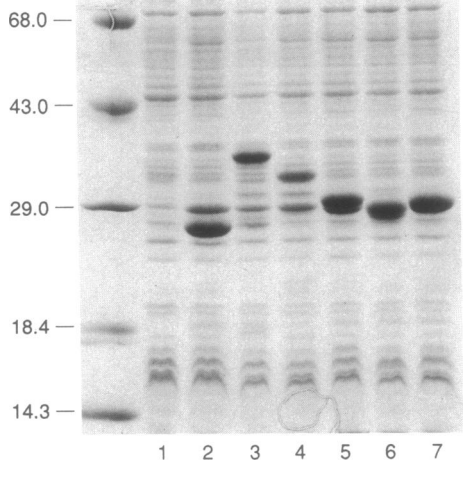

FIG. 2. Synthesis of capsid fusion proteins in *E. coli*. Proteins from *E. coli* cell extracts were separated by SDS/PAGE and stained with Coomassie brilliant blue. Lanes: 1, cell extract (negative control); 2, GST; 3, CAP-A; 4, CAP-B; 5, CAP-1; 6, CAP-2; and 7, CAP-3. Molecular mass markers are shown at left and range in size from 14.3 to 69.0 kDa.

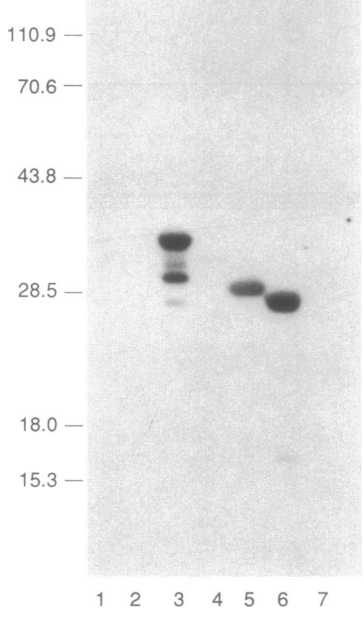

FIG. 3. Immunoblot analysis of capsid fusion proteins. Cell extracts containing the fusion proteins depicted in Fig. 2 were tested for reactivity to NYU 201-6 serum. Lanes: 1, cell extract; 2, GST; 3, CAP-A; 4, CAP-B; 5, CAP-1; 6, CAP-2; and 7, CAP-3. Molecular mass markers are shown at left and range in size from 15.3 to 110.9 kDa.

Table 1. Comparison of CAP-A, CAP-B, and CAP-C seroreactivity

Serum	Type	CAP-A	CAP-B	CAP-C
59-16	Chimpanzee (acute*)	+++	–	–
C-9	Chimpanzee (acute)	+++	–	–
C-10	Chimpanzee (acute)	++	–	–
C-1	Chimpanzee (chronic†)	++	–	–
C-18	Chimpanzee (chronic)	++	–	–
C-19	Chimpanzee (chronic)	++	–	–
C-21	Chimpanzee (chronic)	+++	–	–
C-2	Chimpanzee (chronic)	++	–	–
169-19	Human (acute)	+++	–	–
35-12	Human (acute)	++	–	ND
201-12	Human (acute)	+++	+/–	ND
4-7	Human (acute)	++	–	ND
191-12	Human (acute)	++	–	–

A three-plus alanine aminotransferase serum (+++) indicates that a strong signal was detected by autoradiography after 1–2 hr. A two-plus (++) serum gives rise to a signal of moderate intensity in 2 hr. A single-plus (+) serum gives a strong overnight signal, and a minus (–) indicates no signal was detected after an overnight exposure. ND, not determined.

*Acute serum was taken <12 weeks after onset of alanine aminotransferase abnormality.

†Chronic-phase serum was taken >6 mo after onset of alanine aminotransferase abnormality.

CAP-C. These results suggest that most immunodominant epitopes within the capsid can be found within the first 74 amino acids, as defined by the CAP-A fusion protein.

To further localize the immunodominant epitopes within the amino-terminal region of capsid, an immunoblot analysis of serum cross-reactivity to proteins encoded by plasmids pCAP-1, pCAP-2, and pCAP-3 was done, and the results are summarized in Table 2. Although response to the CAP-1 and CAP-2 polypeptides varied, depending on the anti-serum used, the results show that only one of the sera tested (35-12) cross-reacted with CAP-3. In some cases the response to CAP-A was better than the response to CAP-1 or CAP-2. However, in other cases the CAP-2 signal is as strong, if not stronger, than the CAP-A response. Both CAP-1 and CAP-2 appear to contain reactive epitopes, and the epitope in CAP-2 seems more immunogenic, being recognized by only 16 of the 19 sera. Three sera recognized CAP-A but not CAP-1, CAP-2, or CAP-3. These sera may recognize epitopes that overlap the junctions between CAP-1, CAP-2, and CAP-3.

Table 2. Comparison of CAP-1, CAP-2, and CAP-3 seroreactivity

Serum	Type	CAP-A	CAP-1	CAP-2	CAP-3
C18	Chimpanzee 10 (acute)	+++*	+	+	–
C10	Chimpanzee 194 (acute)	+++	+++	+++	–
59-16	Chimpanzee 59 (acute)	+++	+	+++	ND
59-12	Chimpanzee 59 (acute)	+++	++	+++	–
C9	Chimpanzee 181 (acute)	+++	–	+++	–
213-18	Chimpanzee 213 (acute)	+	+	+	–
C2	Chimpanzee 10 (chronic)	++	–	–	–
C1	Chimpanzee 10 (chronic)	+++	–	–	–
C19	Chimpanzee 10 (chronic)	+++	–	–	–
C4	Chimpanzee 68 (chronic)	+++	+++	+++	ND
169-16	Human (chronic)	ND	+++	+++	–
169-23	Human (chronic)	ND	+++	+++	–
191-12	Human (acute)	++	++	++	–
35-12	Human (acute)	+++	–	+++	+++
201-2	Human (acute)	++	++	++	–
201-5	Human (acute)	++	++	+++	–
201-6	Human (acute)	+++	+++	+++	–
4-5	Human (acute)	+	–	+++	–
4-7	Human (acute)	+++	++	++	–

For definitions of acute and chronic, see legend for Table 1. ND, not determined.

*Immunoblot signal intensities are listed in Table 1.

CAP-A Seroconversion Evaluated by Immunoblot and ELISA. To determine the time interval between HCV infection by transfusion (humans) or inoculation (chimpanzee) and the appearance of an anti-capsid response, the GST–capsid fusion protein encoded by plasmid pCAP-A was used to screen chimpanzee and human seroconversion panels by immunoblot and ELISA. Table 3 shows that 75% of the panels assayed seroconverted to capsid before seroconversion to the C100-3 antigen. The average seroconversion time for C100-3 was 25 weeks (range, 10–62 weeks), whereas the anti-CAP-A response occurs on an average of 8 weeks after infection (range, 2–21 weeks). The results differed for two seroconversion panels: chimpanzee 72 failed to seroconvert to the capsid antigen, and the anti-C100-3 response of human NYU 191 occurred earlier than the anti-capsid response. It was of interest that NYU case 169, which did not develop anti-C100 during 9-mo follow-up, could only be diagnosed as HCV-positive on the basis of a strong response to CAP-A.

DISCUSSION

In this study, a series of GST fusion proteins containing various portions of the HCV capsid region was tested for serologic reactivity against both human and chimpanzee HCV-positive sera. Previously, fusion proteins produced in *E. coli* have been successfully used to map seroreactive epitopes for both structural (13) and nonstructural (19) viral gene products. Fusion proteins that are generally composed of a prokaryotic amino terminus connected to the protein of interest by a sequence that can be recognized and cleaved by a protease have several advantages: they are produced at high levels, are relatively stable, and can easily be purified. For example, GST fusion proteins can be purified by affinity chromatography with glutathione agarose (13), and the GST portion removed by cleavage with the protease thrombin or factor Xa. For producing diagnostic reagents, removal of GST by proteolysis may not be necessary because neither HCV-positive nor normal human serum used in this study significantly cross-reacts with GST alone.

Recently, a peptide composed of capsid amino residues 39–74 and corresponding to a highly hydrophilic region was synthesized (18). This 36-mer oligopeptide, named CP9, was immobilized on a solid support and used in an ELISA assay to detect antibodies against HCV. Anti-CP9 was detected in 68% of patients with sporadic acute non-A, non-B hepatitis and 83% of patients with posttransfusion acute non-A, non-B hepatitis. These results are surprising because the CP9 peptide is similar, although not identical, to the CAP-3 fusion

Table 3. Comparison of the anti-C100-3 and CAP-A seroconversion responses

Seroconversion panel	Peak ALT		Anti-C100-3, weeks*	Anti-CAP-A, weeks
	i.u./liter	Weeks		
Chimpanzee 59	158	13	34	10†
Chimpanzee 213	60	12	18	4†
Chimpanzee 10	223	8	40	2†
Chimpanzee 72	616	17	62	None†
Human NYU 169	317	16	None	14†
Human NYU 201	108	16	16	2*
Human NYU 43	79	6	10	2*
Human NYU 191	336	12	12	21*

All preinoculation and pretransfusion sera were seronegative. i.u., international units. ALT, alanine aminotransferase.

*Results were determined by ELISA.

†Results were determined by immunoblot.

protein that fails to significantly cross-react with most of the HCV-positive sera used in this study. The differences in seroreactivity could be attributed to antigen presentation *in vivo*. Individuals infected with the HCV could potentially generate two different immune responses against capsid, one being anti-peptide response due to major histocompatibility complex-mediated antigen presentation and the other an anti-protein response mediated by B cells and influenced by protein secondary and tertiary structures. In fact, CAP-2, which contains a small peptide-like sequence, cross-reacts with some HCV-positive sera better than CAP-A or capsid protein itself (M.S.N., unpublished results). An alternative explanation for the differences in serologic reactivity between CP9 and CAP-3 could be that a strong immunoreactive epitope exists within the segment of capsid defined by amino acids 60–74. This is the region of CAP-A not covered by CAP-1, CAP-2, or CAP-3. Furthermore, an immunodominant epitope, which begins within CAP-3 and extends beyond amino acid 60, could be destroyed by the elimination of amino acids needed to preserve the structural integrity of this epitope. Clearly, additional mutational analysis is needed to further define the reactive epitopes within this region.

Data presented in Tables 1 and 2 demonstrate that CAP-A fusion protein contains the most immunodominant epitopes and that these epitopes can be localized to the first 40 amino acids of capsid. However, longer exposures of immunoblot strips (2–5 days) revealed that weak cross-reactivity to both CAP-B and CAP-C could be obtained by high-titer sera. Thus, these portions of the capsid protein also contain immunoreactive epitopes.

A comparison of the data from acute- and chronic-phase serum (Table 2) suggests that an anti-peptide-like response indicated by CAP-1 and CAP-2 seroreactivity may be most prevalent during acute infection. When hepatitis progressed to a chronic state, the anti-protein response defined by CAP-A seroreactivity remained, but the anti-peptide response disappeared. By contrast in chimpanzee 68 (Table 2), anti-CAP-1 and CAP-2 responses remained strong 3 yr after HCV infection. Thus, no correlation can be inferred from these data, and additional sera must be tested to evaluate temporal patterns in the response to the different capsid epitopes in different types of infection.

We investigated the time interval between HCV infection and seroconversion to CAP-A. Table 3 summarizes data from both immunoblot and ELISA assays. ELISA is generally considered less specific but more sensitive than immunoblots. In addition, the ELISA format allows for a direct comparison between the anti-C100-3 and anti-capsid immune response. Eighty-six percent of the cases studied seroconverted to capsid before C100-3; mean conversion times were 8 weeks for CAP-A and 25 weeks for C100-3 (Table 3). However, in one case seroconversion to CAP-A was not detected, and in one case the anti-C100-3 response occurred earlier than the capsid response. Thus, although early detection of capsid antibodies reduces the window between HCV infection and serologic detection, other HCV polypeptides will be needed in addition to capsid protein to establish assays capable of detecting 100% of HCV-positive serum.

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