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*J Immunol* 2003; 170:1917-1924; doi: 10.4049/jimmunol.170.4.1917

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**Crystal Structure of a Hydrophobic Immunodominant Antigenic Site on Hepatitis C Virus Core Protein Complexed to Monoclonal Antibody 19D9D6**

Renée Ménez,* Marc Bossus,* Bruno H. Muller,* Geneviève Sibaï,† Pascal Dalbon,† Frédéric Ducancel,* Colette Jolivet-Reynaud,†‡ and Enrico A. Stura2*.

The first crystal structure of a complex between a hepatitis C virus (HCV) core protein-derived peptide (residues 13–40) and the Ab fragment of a murine mAb (19D9D6) has been solved, allowing determination of the recognized epitope and elucidation of its conformation. This Ab, raised against the first 120 residues of the core protein, recognizes core particles and strongly competes with anticore human Abs, suggesting that it is highly representative of the human anti-HCV core response. Its epitope lies within the first 45 aa of the protein, the major antigenic segment of core recognized both by murine and human Abs. Surprisingly, the recognized epitope (29–37: QIVGGYVYL) has an unusual preponderance of hydrophobic residues, some of which are buried in a small hydrophobic core in the nuclear magnetic resonance structure of the peptide (2–45) in solution, suggesting that the Ab may induce a structural rearrangement upon recognition. The flexibility may reside entirely within the Ag, since the Fab’-peptide complex structure at 2.34 Å shows that the Ab binding site is hardly perturbed by complexation. Given that the recognized residues are unlikely to be solvent exposed, we are left with the interesting possibility that Ab-core interactions may take place in a nonaqueous environment. *The Journal of Immunology*, 2003, 170: 1917–1924.

Hepatitis C virus (HCV) is the major causative agent of transfusion-associated hepatitis. It is an enveloped virus with a single-stranded, positive sense RNA genome of ~9600 nucleotides (1) encoding for a polyprotein of 3011 aa, which is then post-translationally cleaved into structural and nonstructural proteins. Among the structural proteins, the core protein is derived from the amino terminus of the viral polypeptide (aa 1–191), and it is thought to form the nucleocapsid of the virion, as its sequence and biological properties resemble those of nucleocapsid proteins from other viruses. The core Ag is highly conserved among the various HCV genotypes (2) and elicits a rapid Ab response after the onset of the disease. Thus, the measurement of HCV core Ab titer in serum is widely used to screen for HCV infection (3). The presence of serum HCV core Ag is associated with active HCV viremia, and its detection is now used for clinical evaluations (4, 5). The HCV core has many effects on cell-host signaling (6, 7), and in chronically infected individuals cellular immune responses against HCV core are severely reduced (8). Thus, the core Ag is an important target for the induction of antiviral immune responses.

Several studies, mainly based on peptide scanning using chemical or recombinant approaches, have clearly established that the first 120 residues of the HCV core protein contain highly immunogenic B cell epitopes that are predominantly recognized by HCV-infected patients (9–13). Indeed, characterization of the sequences recognized by the human Abs raised against this region of the HCV core protein, have provided evidence that the immune response is directed against overlapping linear, but also conformational, epitopes with variable hydrophobic profiles. More precisely, an immunodominant conformational B cell epitope has been identified in the N-terminal region 20–45 of the core protein containing a segment with a high percentage of hydrophobic residues that is likely to be buried within the protein.

To further analyze that HCV-immunodominant region, we generated different mAbs by immunizing mice with the truncated recombinant 1–120 region (9). Among the different anticore mAbs obtained, mAb 19D9D6 appeared to be specific for an epitope encompassed by residues 29–33 within the main antigenic domain (9), a property shared by the major human epitope of core (10, 11). Furthermore, mAb 19D9D6 is of special interest, since it detects, with a good sensitivity, viral core Ag in sera of patients with chronic HCV infection.

To better define the structural characteristics of that immunodominant epitope, we have compared its structure, both free and complexed to mAb 19D9D6. For this purpose we make use of the structures adopted in solution by the core peptide 2–45 (residues 2–45 referred to as S42G in Ref. 9) and compare it to that adopted by the HCV-core peptide 13–40 bound by mAb 19D9D6. Indeed, previous studies, by nuclear magnetic resonance (NMR) and molecular modeling, have allowed characterization of the core peptide 2–45 as consisting of a three-dimensional motif composed of two α-helixes separated by a loop (PDB-ID:1CWX) (14, 15). To characterize the viral core recognition by Abs, we have pursued the

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1 Abbreviations used in this paper: HCV, hepatitis C virus; CDR, complementary-determining region; LVP, lipo-viro-particles; NMR, nuclear magnetic resonance; rmsd, root mean SD.
x-ray crystallographic analysis of the complex between murine Ab 19D9D6 and a 28-aa peptide (residues 13–40 of core) that encompasses the recognized portion within the 2–45 peptide. We report here the structure of this complex at 2.34 Å resolution and compare it with the structure of the free Ab at 1.6 Å. We analyze in detail how the peptide is bound by the Ab and compare it with the longer peptide in solution, placing this structure in the context of other antipeptide Ab complexes. Finally, we discuss the unexpected preponderance of hydrophobic residues in the epitope.

Materials and Methods

Ab production, purification, and sequencing

mAbs were generated by immunizing mice with a truncated recombinant protein corresponding to the immunodominant region (residues 1–120) of HCV nucleocapsid protein (9). They were purified on a protein A-Sepharose 4FF column according to the manufacturer’s instructions (Pharmacia Biotech, Piscataway, NJ). mRNA sequences encoding the light chain and the heavy chain fragments of mAb 19D9D6 were determined by cloning and sequencing of cDNAs. The amino acid sequences of the variable regions are shown in Fig. 1.

Ab fragment generation

The Ab 19D9D6 (IgG1, κ) was produced in vitro and purified as follows. Briefly, the supernatant was diluted 1/2 (v/v) in high salt buffer, then loaded on a protein A column (Pharmacia Biotech) and eluted at pH 6.0, following the manufacturer’s instructions. F(ab’2)2 were obtained by proteolytic cleavage of the IgG by pepsin. Briefly, the purified IgG were mixed with pepsin-agarose in an acidic buffer and digested for 1.5 h at 37°C. The pepsin-agarose was then eliminated by centrifugation, and F(ab’2)2 were recovered in the supernatant. Fab’ were further purified by size exclusion chromatography on a Superdex 200 column (Pharmacia Biotech).

Fab’ were obtained by reducing with 2-ME the most labile disulfide bridges of F(ab’2)2 and by blocking the corresponding free cysteine residue with iodoacetamide. Fab’ were further purified by size exclusion chromatography on a Superdex 200 column (Pharmacia Biotech).

Peptide synthesis

Peptides corresponding to the selected regions of HCV core (Fig. 1) were synthesized on a PE Applied Biosystems (Foster City, CA) automatic synthesizer (model 431A) using fluorenlymethoxycarbonyl and t-buty1 protecting groups and tri fluoroacetic acid deprotection. The peptides were synthesized on a protein A-Sepharose 4FF column according to the manufacturer’s instructions (Pharmacia Biotech, Piscataway, NJ). mRNA sequences encoding the light chain and the heavy chain fragments of mAb 19D9D6 were determined by cloning and sequencing of cDNAs. The amino acid sequences of the variable regions are shown in Fig. 1.

Surface plasmon resonance biosensor analysis

Peptide-Ab interaction analyses were performed on a BIAcore 3000 biosensor optical (BIAcore, Uppsala, Sweden) with simultaneous monitoring of four flow cells. mAb 19D9D6 was immobilized using carbodiimide coupling reagent onto a CMS sensor chip to a final value of 1430 resonance units. Peptide bindings were assessed by passing either peptide 2–45 or peptide 13–40, diluted to concentrations ranging from 0.8–100 nM, over the chip surface with PBS, pH 7.4, containing 0.005% Tween 20 as running buffer. The flow rate was 30 μl/min for 2 min. Sensor data were prepared for kinetic analysis by subtracting the resonance unit value corresponding to an empty reference surface. The association and dissociation data were fitted simultaneously to a single-site binding model using the nonlinear data analysis program Bioevaluation 3.2 (BIAcore, Uppsala, Sweden). The affinity of 19D9D6 for each peptide was calculated as follows: Kd = dissociation rate (koff)/association rate (kon), and was expressed as molarity.

ELISA and inhibition tests

Microtiter plates (MaxiSorp; Nunc, Copenhagen, Denmark) were coated and left for 1 h at 37°C with 100 μl of a solution (0.5 μg/ml in PBS) of the truncated recombinant protein corresponding to the immunodominant region comprising residues 1–120 of HCV nucleocapsid protein. The plates were then blocked at 37°C with 300 μl of PBS with 3% skimmed milk powder and left for 2 h (Regis/Le Plaque, Montault, France). For inhibition experiments, mAb 19D9D6 diluted to 0.15 nM in PBS/0.1% Tween (PBS-T) was preincubated for 2 h at 37°C with different concentrations of synthetic peptides 2–45 and 13–40. A volume of 100 μl of these preincubated solutions was added to each well and incubated for 30 min at 37°C. After several washes, the bound IgG was revealed using peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA), added at a 1/10,000 dilution in PBS-T. After incubation at 37°C for 1 h and after the final washes, a 100-μl volume of 0.03% H2O2 containing ABTS (Sigma-Aldrich, St. Louis, MO) dissolved in 0.1 M citrate buffer (pH 4.3) was added to each well at room temperature. The absorbance at 405 nm was measured 25 min after the start of the reaction using a multichannel spectrophotometer (Asys Hitech, Biochrom, Cambridge, U.K.).

To investigate the behavior of HCV core 1–120 in ELISA and competition ELISA, Ab 19D9D6 was incubated for 2 h with a 2-μM solution of HCV core 1–120. This preincubated solution was then transferred to noncoated plates or to plates coated with core 1–120 and incubated for 30 min.

ELISA with human sera

Wells were coated overnight at 4°C with 100 μl of streptavidin at a concentration of 10 μg/ml in 0.1 M carbonate buffer (pH 9.6) and blocked for 1 h at 37°C with PBS containing 10% goat serum. The plates were then washed three times with PBS containing 0.05% Tween 20 before adding 100 μl of a biotinylated peptide solution (10 μg/ml in PBS) for 2 h at 37°C. After a new wash with PBS-T, 100 μl of serum diluted 1/100 in PBS-T containing 1% goat serum was added and incubated for 2 h at 37°C. The plates were washed again with PBS-T. The secondary Ab, peroxidase-conjugated goat anti-human IgG (Jackson Immunoresearch Laboratories), was then added at a 1/5000 dilution in PBS-T/0.1% goat serum. The plates were incubated 1 h at 37°C and then washed once more with PBS-T. The plates were developed using the commercialized biotin-Merox color kit containing p-ethylthiophenendiamine and hydrogen peroxide. After 10 min of incubation, the plates were read at 492 nm with an ELISA plate reader. The values are the mean OD of triplicate determinations.

Cryocrystallization

Complexes were prepared in solution in advance of the crystalization trials by mixing 40 μl of Fab’ 19D9D6 (6 mg/ml) with 3 μl of peptide (20 mg/ml). Screening for crystallization was conducted by sitting drop vapor diffusion using Q-plates and using multiple drops per reservoir (16, 17), so that the various Fab’ peptide (2–45, 13–40, and 25–45) complexes and the free Fab’ could be tested in parallel over the same precipitant reservoir. The screening and optimization experiments were conducted at 17°C in an air-conditioned room. Only three precipitant solutions derived from other Fab’ crystallization experiments (18–20) were used in the screening with the Fab’ alone and with the peptide complex. The protein drops, ranging in size from 1.2–3.6 μl, were placed on the sitting drop coverglass in the Q-plate setup (16) before layering the precipitant on top without mixing. Different volumes of protein and precipitant drops were used to control the rate of equilibration and the final protein concentration in the drop. Peptides (not related to the Ag) were used in the crystallization of free and complexed Fab’ 19D9D6 during screening and for crystal growth to alter nucleation behavior. The peptide used in crystallization of the Fab’ 19D9D6-peptide complex was EGSDT1TPR1QF1NMWGQ, derived from the sequence of HIV gp120 (residues 414–434) (21), while the peptide used in crystallization of free Fab’ was FYSHGNAEKIV. These two peptides are part of a series of peptides that cause the aggregation of Fab (E. A. Stura, unpublished observations) and hence lower the degree of supersaturation

FIGURE 1. Amino acid sequences of the heavy and light variable chains for Fab’ 19D9D6 and of the core peptides 2–45 and 13–40. The recognized epitope, residues 29–37, is underlined.

19D9D6 Variable Light Chain

1 11 21 31 34 45
OVSQGPPS OASVGSQH ISEK TVSTHE THE GLOQDSQ IGDYTVW
51 61 71 81 82 83 84 85 86 87 88 89 90 91 92
ASSPSSGVPDNEP NQDAGYQDAQ AYTVFQTV QTELELR

19D9D6 Variable Heavy Chain

1 11 21 31 34 44 52 55 56 57 58 59 60 61
QLLLWQGAA LEFLQTVK ECKAVTSST EFDVHGQ EKQGKER EMTPLTV
61 71 72 81 82 83 85 86 88 89 90 91 92 93 94 95
DPGDIDFSL IDELDAZLQ DUECSDKV DQVLFG Qanga CDDVMTYT

HCV-core Peptides

1 14 21 24 31 34 44 45
QDGQPPS OASVGSQH ISEK TVSTHE THE GLOQDSQ IGDYTVW
(2–45); GDSQGIDTLENIK DIQGDNLE EKQGKE
(11–40); GDDQGIDTLENIK DIQGDNLE EKQGKE

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required for nucleation and crystal growth (22). Neither peptide is found ordered in the final crystal structure. Two rounds of crystal improvement by streak seeding followed by macroseeding (23) were added to improve crystal quality.

**Data collection**

Data for Fab’ 19D9D6, complexed and free, were collected from crystals transferred into a cryoprotectant solution containing 27% ethylene glycol, 13.8% (w/w) monomethyl polyethylene glycol 5000, 1.6 mM ZnCl2, 1.6 mM CdCl2, and 55 mM sodium cacodylate (pH 6.5) and flash-frozen in the cryo-stream or in liquid ethane. All data were processed using the HKL package (24). After the second round of optimization by streak seeding, data could be collected from one crystal of Fab’ 19D9D6 complexed with peptide P21 (2 1 2) in the presence of the aggregating peptide FYSHSGNAKQIV of the two crystals are closely related: the ESRF synchrotron facility (Grenoble, France). The unit cell parameters of the HCV core protein on a Rigaku rotating anode x-ray generator with Supper long focusing mirrors. This dataset was collected to 3.4 Å resolution at 100 K on beamline BM10 at the ESRF synchrotron facility (Grenoble, France). There is a single Fab’ 19D9D6 complexed and free, were collected from crystals (The Woodlands, TX) with Supper long focusing mirrors (Natick, MA) under cryogenic conditions as described above. These data were complete and usable to 3.1 Å with a merging R factor of 12.1%. The crystal belongs to the monoclinic space group P21 with unit cell parameters: a = 41.6 Å, b = 104.2 Å, c = 54.1 Å, and β = 91.1°. These data were used for the molecular replacement and in the first stages of refinement to confirm the presence of Ag in the Ab binding site. Subsequently, a second dataset was collected to 2.34 Å resolution at 100 K on beamline BM10 at the ESRF synchrotron facility (Grenoble, France). The unit cell parameters of the two crystals are closely related: a = 42.2 Å, b = 101.7 Å, c = 55.2 Å, and β = 98.7°. The statistics for the second dataset are given in Table I.

The first data collection on a crystal of the unliganded Fab’ 19D9D6 (without the aggregation promoting peptide) was conducted on a Rigaku rotating anode x-ray generator with Supper long focusing mirrors. This crystal diffracted to 3.4 Å resolution, and the data were indexed in space group P21 with cell parameters: a = 75.8 Å, b = 91.6 Å, c = 81.3 Å, and β = 113.5°, and were used in the molecular replacement (20). A second crystal from which data could be collected was found to be: P21 2 1 2 1 2 with cell parameters: a = 60.8 Å, b = 170.8 Å, and c = 40.8 Å, diffracting to 3.9 Å. A third crystal form, strongly related to the second one, was obtained in the presence of the aggregating peptide FYSHSGNAKQIV (F10V): P21 2 1 2 1 2; a = 63.5 Å, b = 173.7 Å, and c = 41.7 Å, and diffracted to 1.6 Å on beamline ID14-E1 at the ESRF synchrotron facility (Grenoble, France). There is a single Fab’ in the asymmetric unit giving a solvent content of 49% (25).

**Structure determination and refinement**

The molecular replacement for Fab’ 19D9D6 has been described in detail previously (20, 26). In brief, the model PDB-ID = 1UCB (27) was used in the molecular replacement using AMoRe (28) in advance of the light and heavy chain sequencing. There is one complex molecule per asymmetric unit, giving a solvent content of 47% (25). With the availability of the sequences, a FASTA search was conducted against the PDB database and the heavy and light chains replaced by those from PDB-ID = 1DBA (29) and PDB-ID = 1HIL (30), respectively. The sequences were then modified to conform to those determined for Fab’ 19D9D6. Several crystals were grown and analyzed, and data were collected. Each new dataset was solved by molecular replacement using AMoRe with the best refined model of Fab’ 19D9D6 available at the time. The molecular replacement was followed by rigid body refinement and cycles of conjugate gradient and anisotropic temperature factor refinement using CNS (31) with the maximum likelihood protocol. Progress was judged by the decrease in the free R value. Electron density maps (omit weight-weighted 2Fo-Fc and Fo-Fc) were calculated and displayed using the XnVView suite of programs (32) and Turbo (33).

In the refinement of the Fab’ 19D9D6 in complex with the peptide, the complementary-determining regions (CDR) of the Ab were removed to avoid model bias in the region of the Ag binding site. The combining site region was then rebuilt following structure refinement. In the final electron density map, the CDR loops are all well defined. Although density corresponding to the peptide had been seen earlier, the peptide was not modeled until all the Fab’ had been built correctly. The density was first fitted by a chain of alanines, and the side chains were later positioned when the density for them was clear. The refinement of the free Ab followed the same protocol as that for the complex.

**Table I. Crystal data and refinement statistics**

<table>
<thead>
<tr>
<th></th>
<th>Free 19D9D6</th>
<th>19D9D6 Complex with P13–40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data collection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>20–1.6</td>
<td>20–2.34</td>
</tr>
<tr>
<td>Total observations</td>
<td>47973</td>
<td>17893</td>
</tr>
<tr>
<td>Uniq. reflections</td>
<td>42445</td>
<td>14779</td>
</tr>
<tr>
<td>Rsym (%)</td>
<td>8 (22°)</td>
<td>6.7 (23.3°)</td>
</tr>
<tr>
<td>Completeness</td>
<td>79.5 (42°)</td>
<td>98.8 (91.3°)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>4.5</td>
<td>3</td>
</tr>
<tr>
<td>I/σ</td>
<td>13.2 (1.5°)</td>
<td>9.2 (1.5°)</td>
</tr>
<tr>
<td>Space group</td>
<td>P21 2 1</td>
<td></td>
</tr>
<tr>
<td>Unit cell parameters (Å, °)</td>
<td>a = 63.48; b = 173.68; c = 41.73</td>
<td>a = 42.18; b = 101.7; c = 55.18; β = 98.6</td>
</tr>
<tr>
<td>Vm (Å³/Da)</td>
<td>2.41</td>
<td>2.33</td>
</tr>
<tr>
<td>Solvent content (%)</td>
<td>49</td>
<td>47</td>
</tr>
<tr>
<td>Refinement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total residues</td>
<td>438</td>
<td>454</td>
</tr>
<tr>
<td>No. of water molecules</td>
<td>202</td>
<td>202</td>
</tr>
<tr>
<td>Refinement range (Å)</td>
<td>20–1.6</td>
<td>20–2.34</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>18.1 (20.0)</td>
<td>19.0 (21.6)</td>
</tr>
<tr>
<td>Rwork (%)</td>
<td>21.1 (23.5)</td>
<td>24.5 (26.8)</td>
</tr>
<tr>
<td>Bond length rmsd (Å)</td>
<td>0.0056</td>
<td>0.0065</td>
</tr>
<tr>
<td>Angles rmsd (°)</td>
<td>1.45</td>
<td>1.40</td>
</tr>
<tr>
<td>Residues in most favored regions (%)</td>
<td>91.3</td>
<td>87.1</td>
</tr>
<tr>
<td>Residues in disallowed regions (%)</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Average B value (Å²)</td>
<td>22.92</td>
<td>36.44</td>
</tr>
</tbody>
</table>

* Values for outer shell (free Fab’, 1.66–1.80 Å; peptide complex, 2.39–2.34 Å).

* For F > 2σ(F) (all reflections F > 0).
Results

Immunological properties of core peptide 13–40

Crystallization trials with core peptides 2–45 and 25–45 yielded no crystals, probably because these peptides suffer from aggregation problems when used in excess to the Fab’. Thus, a shorter peptide, 13–40, was designed. While still containing the recognized epitope (9), with similar Ag-binding equilibrium dissociation constants ($K_d$) as 2–45 (Table II), this peptide has better crystallization properties and has allowed us to obtain crystals of the Fab’ 19D9D6-peptide complex. To demonstrate that the peptide 13–40 also possesses valuable immunological properties, competition ELISA experiments were conducted between immobilized core 1–120 and peptides 2–45 or 13–40 (Fig. 2). With the titer plate coated with core 1–120, both peptides are equally effective inhibitors in the range $10^{-10}$–$10^{-7}$ M. In addition, at higher concentrations the shorter peptide acts as an effective competitor and gives a classical complete inhibition curve, while peptide 2–45 shows a nonstandard behavior that could be explained by partial aggregation at high concentrations. This anomalous behavior at high concentrations is also shared by core 1–120. Thus, peptide 13–40 appears not to suffer from the aggregation problems found for 2–45 and core 1–120, but is an equally effective or better competitor than the original Ag and the longer peptide.

The recognition of peptide 13–40 by HCV-positive sera has been analyzed to determine whether, like 2–45, this peptide is recognized by human sera (Fig. 3). Peptide 13–40 was tested against HCV-positive sera that had been shown to be immunoreactive with peptides corresponding to residues 2–45 (S42G), 25–45 (V22G), and 12–35 (K22Y) from the sequence of HCV core (name in parentheses corresponds to the code in Ref. 9).

These results provide a strong support to the structural results reported here. Indeed, the core peptide corresponding to residues 13–40 is recognized by 19D9D6 with an affinity as strong as 2–45 in BLACore and shows a classical and specific behavior in competition ELISA against core 1–120 against which it was raised. Finally, the shorter peptide is equally well recognized by human HCV core-positive sera that recognize an immunodominant epitope on both 2–45 and 25–45. Together these results clearly establish that the HCV-core peptide 13–40 mimics the immunological properties of the longer peptide 2–45 and is thus a valuable tool to study how 19D9D6 specifically recognizes the immunodominant epitope comprised within its sequence.

Structure of the 19D9D6 Fab’ 13–40 peptide complex

The final electron density maps of the complex between the 19D9D6 Fab’ and the 13–40 peptide, at 2.34 Å resolution, allows the positioning of all the 438 residues of the Fab’ (218 from the light chain and 220 from the heavy chain), 15 of the 28 peptide residues, and 202 ordered solvent molecules. The refinement statistics are given in Table I. All residues are within the allowed regions of the Ramachandran plot (PROCHECK), with the exception of Ala L51, as is commonly found in most L2 hypervariable loops. The only regions of the Fab’ in weak density are those that are often disordered in other Fab structures.

Table II. Anticore mAb 19D9D6 affinity constants for peptides 2–45 and 13–40

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$k_{on}$ M$^{-1}$ s$^{-1}$</th>
<th>$k_{off}$ s$^{-1}$</th>
<th>$K_d$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2–45</td>
<td>$1.06 \pm 0.01 \times 10^6$</td>
<td>$2.02 \pm 0.01 \times 10^3$</td>
<td>$1.91 \pm 0.02$</td>
</tr>
<tr>
<td>13–40</td>
<td>$2.05 \pm 0.02 \times 10^6$</td>
<td>$2.65 \pm 0.04 \times 10^3$</td>
<td>$1.3 \pm 0.1$</td>
</tr>
</tbody>
</table>

$k_{on}$ and $k_{off}$ correspond to association rate and dissociation rate respectively. $K_d = k_{off}/k_{on}$

The Ab binding site is a crevice, as has been observed for other Ab-peptide complexes. The surface areas buried upon binding are 592 and 648 Å$^2$, on the Fab’ and the peptide, respectively, calculated using a 1.4Å probe in the program AREAIMOL. This represents the loss of accessibility of the surface to water molecules. These figures are comparable to other Fab-peptide complexes (40).

The peptide is bound between the $V_L$ and $V_H$ domains, interacting primarily with the hypervariable loops L1, L3, H1, H2, and H3, but not with L2. Nonpolar interactions dominate, and only the QIVGG stretch interacts via hydrogen bonds with the hypervariable regions of the Fab’ (Fig. 4). Surprisingly, given the central and dominant role classically played by the CDR-H3, only two residues from the H3 loop are involved in peptide interactions, and of these, only Gln H99 plays a central role in peptide binding. Thus, the peptide binding contribution of the heavy chain (52%) is comparable to that of the light chain. The relatively important role of the light chain is mainly a function of the long CDR-L1. The conformation of the bound peptide is best described as a wide bend with the VGG segment buried deepest in the Ag-combining site. Only residues 29–37 (QIVGGVYLL) of the 13–40 peptide interact with mAb 19D9D6 and are well ordered in electron density. The other six residues of the peptide that can be modeled are more flexible and are not part of the recognized epitope. Of these, arginine residues P39 and P40 at the C terminus of the peptide are stabilized through crystal contacts, while Pro P25 and the three

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Competition ELISA between peptides. The inhibition curve for 13–40 (continuous line) shows that the shorter peptide out-competes the longer one (2–45; broken line) by a factor of 10–50. The two peptides have a similar behavior at low peptide concentrations (in the range $10^{-12}$–$10^{-9}$), but at higher peptide concentrations, 2–45 is a less effective competitor than 13–40, possibly due to some degree of aggregation (to be verified by other techniques) of the 2–45 peptide.

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Immunoreactivity of anticore human sera against 13–40 tested by ELISA with sera diluted to 1/100. All 15 anti-HCV core-positive sera (S1–15) are above the threshold (0.037) calculated from the mean of the four negative anti-HCV core sera (T1–4) plus 3 SD.
glycines, P26, P27, and P28, make only minor van der Waals contacts with residues from the H1 and H2 CDR loops.

Twelve Ab positions from the \( V_L \) domain and 10 from the \( V_H \) are involved in the complex formation (Table III). The peptide makes a total of 134 contacts (\( \leq 4 \) Å), among which 10 are hydrogen bonds, five each with the \( V_L \) and \( V_H \) domains (Table IV). Within the epitope, four H bonds are from main chain to main chain atoms, five are from side chain to main chain, and one is formed between side chains (Table IV). The first residue in interaction with the Fab is Gln P29, contacting the amide atoms of Ser H33 and Thr H52A through a bifurcated hydrogen bond from its Oe1 atom. Its Nε2 atom is within hydrogen bond distance of Thr H52A Oγ1 and the amide of Glu H53. Val P31 is in a prominent position to make extensive van der Waals interactions with residues from the L3 and from each of the three Fab heavy chain hypervariable loops. The amide of Gly P32 makes a hydrogen bond to the Oε1 Gln H99, a key residue from the Fab CDR-H3 that plays an important role in stabilization of the IVGG stretch of the peptide as
well as interacting with Tyr P35. Tyr P35 is a key residue of the hydrophobic binding core that includes Val P34, Leu P36, and Ile P30 and is the central member of the VYL motif that is structurally conserved in solution (14). Val P34 becomes sandwiched between this tyrosine and Tyr L92 from L3. The hydrophobic core is extended to Val P31 through the mediation of Pro L94, Leu L96, His H35, Trp H50, and Gln H99, all common contact residues to both Tyr P35 and Val P31.

The free Fab’ and the HCV-peptide complex crystallize in different crystal forms, and their crystal contacts are different, but they superpose well (mean root SD (rmsd): V H, 0.58 Å; V L, 0.49 Å; V H \(\times\) V L, 1.59 Å; V H, 0.41 Å) and even their elbow angles are virtually identical. All the V H side chains involved in peptide binding conserve their orientation in the native as in the complex, and a detailed comparison of the free and complexed 19D9D6 Fab structures shows that only minor structural adjustments are needed in the V L domain to accommodate the peptide. Some of these changes involve the long flexible CDR-L1 segment (L27a-f to L29; Fig. 1). Because of its flexibility, it responds equally well to changes in its crystal environment or complexation status. The side chain conformation of Arg L27 is different in the HCV core peptide complex, the uncomplexed Fab’, and the complex with *P. magnus* protein L mutant D55A (without a bound Ag) (26). Thus, this difference is not clearly attributable to peptide binding. However, the movement of the side chain of Asn L27d, which in the complex makes a bifurcated hydrogen bond through its N6 with the carboxyl oxygen atoms of Gly P32 and Leu P35 of the peptide, should be attributed to peptide binding. In addition, we mention the shift of the segment L92–95 of L3 by up to 0.6 Å relative to the heavy chain, with retention of conformation. This movement slightly opens the binding site to better accommodate the peptide. Together these observations not only confirm the flexibility of that particular segment of CDR L1, but also that these conformational changes are induced by peptide binding.

**Epitope conformation in solution and Ab bound**

The conformation of peptide 2–45 has been characterized in solution (PDB-ID: 1CWX, 14, 15). It folds into two helices that pack against each other, masking with their interaction a small hydrophobic core. This structure is different from that adopted when bound to mAb 19D9D6. In the crystal structure, the peptide conformation is stabilized by interactions with the Ab, by intrapeptide hydrogen bonds and several van der Waals interactions (Tables III–IV). The five intrapeptide hydrogen bonds are from main chain atoms to main chain atoms (Table IV). The carbonyl from Pro P25 contacts the amide of Gly P27, whose carbonyl, in turn, connects to the amide of Gln P29. The carbonyl of Gly P32 is within hydrogen bonding distance of the amides of Val P34 and Tyr P35, and the network of stabilizing hydrogen bonds is completed by Gly P33, whose carbonyl contacts the amide of Leu P36. Furthermore, the side chains of Ile P30, Val P34, Tyr P35, and Leu P36 participate in the creation of a hydrophobic core that stabilizes the peptide conformation and favors peptide-Ab interaction. In comparison with the solution structure, only a short stretch of five residues (32–26: GGYYLY) is maintained. The best agreement gives an rmsd of 0.78 over all backbone atoms and 0.84 over all atoms against model 1CWX:3 (rmsd of 3.1 over the whole epitope 29–37 over backbone atoms; 3.6 over all atoms). Finally, we have analyzed the likelihood that the epitope within the HCV-core peptides 13–40 and 2–45 is buried within a hydrophobic core, as suggested by the solution structure, and have compared the values obtained with those for sequences recognized by other Abs complexed to bacteria- or virus-derived peptides whose structures have been solved. For this purpose, we used the PROTScale program with the Chothia (35) or Janin (36) scales. The result is that the epitope recognized by mAb 19D9D6 has a high probability of being buried. The values obtained with our peptide surpass all the values.

**Table III. Peptide residues involved in van der Waals contacts**

<table>
<thead>
<tr>
<th>Peptide Residues</th>
<th>Fab’ Contact Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro P25</td>
<td>Asp H31</td>
</tr>
<tr>
<td>Gly P26</td>
<td>Thr H30, Asp H31, Glu H53</td>
</tr>
<tr>
<td>Gly P27</td>
<td>Asn H52, Glu H53</td>
</tr>
<tr>
<td>Gln P29</td>
<td>Phe H32, Ser H33, Trp H50, Val H51, Asn H52, Thr H52a, Glu H53</td>
</tr>
<tr>
<td>Ile P30</td>
<td>Gln H99</td>
</tr>
<tr>
<td>Val P31</td>
<td>Pro L94, Leu L96, His H35, Trp H50, Gln H99</td>
</tr>
<tr>
<td>Gly P32</td>
<td>Ala L91, Tyr L92, Gln H99</td>
</tr>
<tr>
<td>Gly P33</td>
<td>Ala L91, Tyr L92, Asn L27d, Tyr L32, Gln H99</td>
</tr>
<tr>
<td>Val P34</td>
<td>Tyr L92, Asn L27d</td>
</tr>
<tr>
<td>Tyr P35</td>
<td>Pro L94, Leu L96, His H35, Trp H50, Gln H99</td>
</tr>
<tr>
<td>Leu P36</td>
<td>Asn L27d, Thr L28, Tyr L32, Leu H97</td>
</tr>
<tr>
<td>Leu P37</td>
<td>Thr L28</td>
</tr>
<tr>
<td>Pro P38</td>
<td>Arg L27f</td>
</tr>
</tbody>
</table>

**Table IV. Peptide hydrogen bond interactions**

<table>
<thead>
<tr>
<th>Peptide Atom</th>
<th>Contact Atom</th>
<th>H Bond Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide interactions with heavy chain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gln P29 Oε1</td>
<td>Ser H33 N</td>
<td>2.93</td>
</tr>
<tr>
<td></td>
<td>Thr H52a N</td>
<td>3.15</td>
</tr>
<tr>
<td>Gln P29 Ne2</td>
<td>Thr H52a Oyl</td>
<td>2.73</td>
</tr>
<tr>
<td></td>
<td>Glu H53 N</td>
<td>2.84</td>
</tr>
<tr>
<td>Gly P32 N</td>
<td>Gln H99 Oε1</td>
<td>2.84</td>
</tr>
<tr>
<td>Val P34 O</td>
<td>Ser H186 O2b</td>
<td>2.52</td>
</tr>
<tr>
<td>Tyr P35 O</td>
<td>Ser H134 O2b</td>
<td>2.64</td>
</tr>
<tr>
<td>Peptide interactions with light chain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gly P33 N</td>
<td>Ala L91 O</td>
<td>2.83</td>
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<tr>
<td></td>
<td>Tyr L92 O</td>
<td>3.22</td>
</tr>
<tr>
<td>Gly P33 O</td>
<td>Asn L27d N82</td>
<td>2.94</td>
</tr>
<tr>
<td>Val P34 N</td>
<td>Tyr L92 O</td>
<td>2.90</td>
</tr>
<tr>
<td>Leu P36 O</td>
<td>Asn L27d N82</td>
<td>2.90</td>
</tr>
<tr>
<td>Arg P39 NH1</td>
<td>Ser L7 Oylb</td>
<td>2.95</td>
</tr>
<tr>
<td>NH2</td>
<td>Ser L7 Oylb</td>
<td>2.93</td>
</tr>
<tr>
<td>Peptide residues interacting with water molecules</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gln P29 O</td>
<td>Val P31 O</td>
<td></td>
</tr>
</tbody>
</table>

* Distances calculated using Contact from the CCP4 program suite.

* Contact to a symmetry-related molecule in the asymmetric unit.
the Ab-bound peptide is 478 Å² of solvent-accessible surface buried in peptide-peptide interactions in the NMR structure of the peptide compared with the Fab-bound conformation. Taking this into account, the difference in the NMR structure of the peptide compared with the nes QIVGGVYLL (core 29–45) as the minimal linear epitope for mAb 19D9D6, consistent with its role of changing the Ag conformation.

Discussion

The structure of the complex defines QIVGGVYLL (core 29–37) as the minimal linear epitope for mAb 19D9D6, consistent with its recognition of peptides 2–45 and 25–45, but not of 12–35, which lacks the last two residues of the epitope (9). Other segments of the core protein could come in contact with mAb 19D9D6 when complexed to the 1–120 polypeptide against which the Ab was raised or to the native core particle, which is also well recognized by this mAb. Given that this is the first crystal structure of an HCV core Ag, it is not possible at present to state that the recognized epitope is purely linear. The comparison with the NMR structure of the peptide (14, 15) shows that apart from a short central stretch (GGVYL; Fig. 4) the Ab induces a particular conformation of the bound peptide that is different from that adopted by the Ag alone. This is reminiscent of the situation observed in the complex of Fab C3 and a poliovirus-derived peptide, where the four central residues of the epitope adopt the same conformation on the viral capsid as in the Ab binding site, but the flanking sequences are substantially distorted. In our case we do not know whether the conformation of this segment on the nucleocapsid is closer to that of the peptide in solution or to that recognized by mAb 19D9D6. The PROTSISE sequence analysis suggests that it is likely that this epitope could be partially buried in the protein hydrophobic core and thus is unlikely to be presented in an aqueous environment, implying that the Ab would still be required to induce changes in the protein to bind. On the other hand, it is not conceivable that such a conformation may be found exposed in the lipidic environment of the nucleocapsid and within the emulsion used in the immunization, where the core protein (1–120) fused to glutathione-S-transferase was emulsified with an equal volume of CFA. In such an environment the hydrophobic conformation between the two helices of the peptide, as observed in the NMR structure (14, 15) where most of the large side chains are buried, is likely to be destabilized and is more likely to be presented to the immune system in the more open structure recognized by mAb 19D9D6.

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Nonetheless, in vitro this Ab is capable of binding with high affinity the Ag in solution without detergents, oils, or lipids, showing that the hydrophobic environment of its binding site is sufficient to destabilize the peptide structure. It is easy to envisage a conformational change where the two sequential glycines might act as the hinge around which the conformation is altered (Fig. 4). The facts that the Ag-combining site remains unchanged on binding and that the glycines are buried deeply at the center of the site support the idea that the Ab binding site may be well adapted for its role of changing the Ag conformation.

In Table V a strong discrepancy is demonstrated between the low 0.277 value on the Chothia scale and the high 7.5 value on the Janin scale for the hemagglutinin HA1 peptide bound by Fabs 17/9 (30) and 26/9 (PDB-Id: 1HIM, 1FRG). This is interesting because this epitope is solvent exposed, but hidden within the hemagglutinin trimer, and becomes exposed only after the low pH-induced fusogenic transition, a process that can be inhibited by certain Abs; the crystal structure of one of these has been recently solved (PDB-Id: 1KEN) (36). This example highlights the fact that many viral proteins have some inherent flexibility that permits conformational changes to occur. Flexibility may explain the discrepancy between the two scales and suggest that for flexible polypeptides it is difficult to draw structural conclusions from the primary sequence. Although the QIVGGVYLL epitope is flexible, the strong agreement between the two scales and the unusually large values attained, which surpass any stretch previously found within the sequence of any bacterial or viral Ag, leave no doubt of the strong likelihood of this epitope being buried in a protein core or exposed in a nonaqueous environment.

The ELISA results support the idea that the HCV core and the 2–45 fragment are prone to aggregation. A comparison of the results obtained on core peptides studied by competitive ELISA (Fig. 2) shows that the shorter peptide (13–40) is a more effective competitor that the longer one (2–45), while it would be expected that both peptides should function in a similar manner, since both peptides contain the entire recognized epitope. This is true only at low concentration, but as the concentration is increased, the longer

Table V. Peptide hydrophobicity/buried in protein core

<table>
<thead>
<tr>
<th>PDB Code</th>
<th>Peptide Sequence</th>
<th>Chothia Scale</th>
<th>Janin Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV core</td>
<td>RNTNRRFPQDV KFPGGQIVGGVYLLFRR</td>
<td>0.403</td>
<td>10.6</td>
</tr>
<tr>
<td>1A3R</td>
<td>VKAEVNLNPDLQPTTE</td>
<td>0.263</td>
<td>5.9</td>
</tr>
<tr>
<td>1ACY</td>
<td>YNKRKHGPRTGRAFYTTKN IIGC</td>
<td>0.351</td>
<td>6.9</td>
</tr>
<tr>
<td>1BOG</td>
<td>GATPREDINQKL</td>
<td>0.278</td>
<td>6.8</td>
</tr>
<tr>
<td>1CU4</td>
<td>APKTNMKIMA</td>
<td>0.254</td>
<td>4.3</td>
</tr>
<tr>
<td>1FPT</td>
<td>CVIMTVDNAPSTNKKD</td>
<td>0.375</td>
<td>6.4</td>
</tr>
<tr>
<td>1FRG</td>
<td>DVPDYASL</td>
<td>0.277</td>
<td>7.5</td>
</tr>
<tr>
<td>1GI</td>
<td>CKRIHIGPRAFYTC</td>
<td>0.351</td>
<td>6.9</td>
</tr>
<tr>
<td>1SM3</td>
<td>TSAPDTRAPPST</td>
<td>0.264</td>
<td>7.3</td>
</tr>
<tr>
<td>ITET</td>
<td>VEVPQGQHDQSQKKA</td>
<td>0.318</td>
<td>6.9</td>
</tr>
<tr>
<td>12HRP</td>
<td>MSLPGKMKPK</td>
<td>0.236</td>
<td>4.6</td>
</tr>
</tbody>
</table>

*Maximum value obtained for each of the peptides analyzed. Values were obtained using PROTSISE (www.expasy.ch). The larger the value, greater the probability that the stretch may be buried inside the core of the protein from which the peptide was derived.*
peptide may aggregate and thus become less effective, a behavior shared by the core 1–120.

The unplanned result of this study is the determination of a shorter, more soluble fragment (13–40) for diagnostic use, as it does not have the aggregation problems of either the whole 1–120 or 2–45 core segments.

In conclusion, the structure of the complex of Fab’ 19D9D6 with core peptide 13–40 offers an unique opportunity to understand the actions of Abs, not just in solutions but also against Ags that might be presented in lipidic environments. Given that this monoclonal competes effectively with human sera, it is suggested that human anti-HCV Abs may share many of the features of mAb 19D9D6, including the ability to destabilize, partially at least, the structure of the Ag and/or recognized Ags that are associated with a lipidic phase.

Acknowledgments
We thank the local contacts from beamlines ID14-EH21 and BM30 at the European Synchrotron Radiation Facility. We also thank Dr. Loic Martin for performing the BIAcore experiments, Dr. Kunchithapadam Swaminathan (National University of Singapore) for help refining this Ab-peptide complex, and Dr. Dominique Rolland for biochemical help. We are grateful to Dr. François Penin for helpful discussion.

References