Characterization of Mimotopes Mimicking an Immunodominant Conformational Epitope on the Hepatitis C Virus NS3 Helicase

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The hepatitis C virus (HCV) nonstructural 3 (NS3) protein is composed of an amino terminal protease and a carboxyl terminal RNA helicase. NS3 contains major antigenic epitopes. The antibody response to NS3 appears early in the course of infection and is focused on the helicase region. However, this response cannot be defined by short synthetic peptides indicating the recognition of conformation-dependent epitopes. In this study, we have screened a dodecapeptide library displayed on phage with anti-NS3 mouse monoclonal antibodies (mAbs) that compete with each other and human anti-HCV NS3 positive sera. Two peptides (mimotopes) were selected that appeared to mimic an immunodominant epitope since they were recognized specifically by the different anti-NS3 mAbs of the study and by human sera from HCV infected patients. Homology search between the two mimotopes and the NS3 sequence showed that one of the two peptides shared amino acid similarities with NS3 at residues 1396–1398 on a very accessible loop as visualized on the three-dimensional structure of the helicase domain whereas the other one had two amino acids similar to nearby residues 1376 and 1378. Reproduced as synthetic dodecapeptides, the two mimotopes were recognized specifically by 19 and 22, respectively, out of 49 sera from HCV infected patients. These mimotopes allowed also the detection of anti-NS3 antibodies in sera of HCV patients at the seroconversion stage. These results suggest that the two NS3 mimotopes are potential tools for the diagnosis of HCV infection. J. Med. Virol. 72:385–395, 2004. © 2004 Wiley-Liss, Inc.

KEY WORDS: NS3 helicase; monoclonal antibody; HCV human response; phage-displayed peptide library; mimotopes

INTRODUCTION

Hepatitis C virus (HCV) is currently the main etiological agent of posttransfusion non-A, non-B hepatitis. The viral genome is a positive-stranded RNA of approximately 9.5 kb and has one large open reading frame that encodes for a polyprotein of 3,011 amino acids [Choo et al., 1989; Houghton et al., 1991]. This precursor is then cleaved posttranslationally into putative structural (core, envelope proteins E1 and E2) and nonstructural proteins (NS2, NS3, NS4, NS5). The NS3 protein, which is one of most conserved proteins of HCV, contains enzymatic domains that are likely to play a pivotal role in the virus life cycle. The N-terminal one-third contains a serine-protease domain that is responsible for the processing of the HCV nonstructural polyprotein [Bartenschlager et al., 1993] whereas the C-terminal two-thirds corresponds to an adenosine triphosphatase (ATPase)/helicase [Suzich et al., 1993; Jin and Peterson, 1995; Neddermann et al., 1997], an enzyme capable of unwinding duplex RNA. The HCV NS3 helicase is involved in modulation of RNA structure during replication. Its structure has been solved by crystallography with a 2.1 Å resolution [Yao et al., 1997; Kwong et al., 2000].
Previous data, which used recombinant protein fragments, indicated that NS3 had the main B-cell epitopes in HCV infection [Van der Poel et al., 1991; Sällberg et al., 1996; Ou-Yang et al., 1999] and clinical studies demonstrated that antibodies to NS3 appeared early in the course of infection, usually before or concomitantly with seroconversion to anti-core [Van der Poel et al., 1991]. The antibody response in both infected humans and immunized mice were almost exclusively focused on the ATPase/helicase [Chen et al., 1998]. However, this response could not be defined by short synthetic peptides, suggesting that the response was directed at conformation-dependent epitopes [Mondelli et al., 1994; Claeyss et al., 1995; Chen et al., 1998].

Serodiagnosis of HCV is based on the use of recombinant proteins and/or peptides corresponding to various regions of Core, NS3, NS4, and NS5. On that basis, the localization of the immunodominant epitopes of HCV proteins and, more particularly, of NS3, is important for the construction and the development of specific and sensitive HCV immunoassays.

For this reason, mouse monoclonal antibodies raised against a NS3 recombinant protein in the helicase domain were used to screen a dodecapeptide phage library displayed on phage. Random peptides libraries displayed on phage have proved to be effective tools for research in immunology [Scott and Smith, 1990; Cortese et al., 1994; Deroo and Muller, 2001]. Indeed, this technology has been used to determine the target sequences for monoclonal antibodies [Michel et al., 1999; Jolivet-Reynaud et al., 2001] and to identify ligands for antibodies in complex polyclonal sera [Folgari et al., 1994], synovial and cerebrospinal fluids [Cortese et al., 1996; Jolivet-Reynaud et al., 1999]. The identification of HCV epitopes has been made using this approach with human sera [Prezzi et al., 1996; Bartoli et al., 1998; Pereboeva et al., 1998; Minenkova et al., 2001]. However, only one study described the use of antigenic and peptide, respectively 

**MATERIALS AND METHODS**

**Human Sera**

HCV positive sera were obtained from the Laboratoire d’Hygiène et de Virologie, (Hôpital Edouard Herriot, Lyon, France) and sera from healthy individuals were from the Établissement de Transfusion Sanguine, (Lyon, France). These sera were remnants of samples submitted for routine diagnostic testing. Anti-HCV seroconversion were from Boston Biomedica, Inc. (Bridgewater, MA). All sera were tested by second generation anti-HCV ELISA (Abbott Laboratories, Abbott Park, IL) and third generation recombinant immunoblot assay, RIBA (Chiron Corporation, Emeryville, CA).

**Cloning, Expression, and Purification of NS3 Recombinant Proteins**

HCV RNA from serum samples were extracted and reverse-transcribed as described previously [Li et al., 1992]. Amplification of cDNA representing a part of the NS3 protein coding region (amino acids 1193–1464) of HCV 1a genotype was carried out by polymerase chain reaction (PCR) using primers corresponding to the 5’ and 3’ end of either the 1193–1464 or 1372–1464 NS3 protein coding gene plus BamH1 and EcoR1. PCR products were then cloned into the expression plasmids pGEX-3 (Pharmacia, France) or pET-21b (Novagen, UK), both double digested with restriction enzymes BamH1 and EcoR1. The resulting plasmids, pGE-NS3 and pET-NS3, encode the HCV truncated NS3 proteins fused to a glutathione-S-transferase (GST) protein [Smith and Johnson, 1988] and to a hexahistidine peptide, respectively [Arnold, 1991]. These different fused proteins were called NS3(272)-GST, NS3(93)-GST and NS3(272)-His, NS3(93)-His, respectively.

**Monoclonal Antibodies**

BALB/c JYco female mice, 4- to 6-weeks-old (IFFA Credo, Les Oncins, l’Arbresle, France) were immunized by intraperitoneal injection with 15 μg of purified NS3(272)-GST, emulsified with an equal volume of Freund’s complete adjuvant. Three other injections were then made, using incomplete adjuvant every 2 weeks. Four days after the last injection, spleen cells were harvested and fused according to Köhler and Milstein [1975, 1976] with the Sp 2/0-Ag14 mouse myeloma cell line. After 12–14 days, the culture supernatants were screened with an ELISA in which the solid phase was coated with the antigen used for immunization. Positive colonies were subcloned twice by limiting dilution. Ascitic fluids were obtained from mice primed with a 0.5 ml intraperitoneal injection of Pristane and then injected with 10^6 hybridoma cells. IgG antibodies were purified on a protein A-Sepharose 4PF column according to the instructions of the manufacturer (Pharmacia, Uppsala, Sweden).

**Biotinylation of Monoclonal Antibodies**

Purified mAbs were biotinylated using Sulfo-NHS-LC-Biotin (Merck, Rockford, IL) according to Gretch et al. [1987].

**Indirect ELISA**

Ninety-six well plates Maxisorb (Nunc, Denmark) were coated with 100 μl/ well of peptide or recombinant
ELISA Competition of Monoclonal Antibodies With Themselves and Anti-NS3 \(^{+}\) Human Sera

The plates were coated with the recombinant protein NS3(272)-His, blocked and washed as described above. Hundred microliters of the first antibody diluted in PBS-T containing 10% goat serum (mAb at the final concentration of 2 \(\mu\)g or anti-NS3 human sera diluted to 1:100), were then added and incubated overnight at 4°C. After three washings, the second mAb which has been previously biotinylated was added at the final concentration of 10 nM and incubated for 1 hr at 37°C. After a new wash with PBS-T, the secondary antibody, peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), was then added at a 1:5,000 dilution in PBS-T-goat serum. The plates were incubated for 1 hr at 37°C and then washed once more with PBS-T. The plates were developed using the commercialized bioMérieux Color kit (Marcy l’Etoile, France) containing ortho phenylene diamine and hydrogen peroxide. After 10 min of incubation, the reaction was stopped with 1.8 N H\(_2\)SO\(_4\) and the plates were read at 492 nm, using an ELISA plate reader. The values are expressed as the mean OD of triplicate.

DNA Sequencing

Single-stranded DNA was prepared from the purified phages as described by Sambrook et al. [1982]. The nucleotide sequence of the gene III insert was determined according to Sanger’s modified method [Sanger et al., 1977] with an Applied Biosystems DNA sequencer (Model 377A) using the BigDye™ terminator cycle sequencing ready reaction kit (Applied Biosystems, Warrington, UK). Cycle sequencing was undertaken with a primer 5’ HO-CCCTCATAGTTAGCGTAACG-3’ corresponding to the pIII gene sequence. The amino acid sequence of the insert was deduced from the nucleotide sequence.

Phage Peptide Library

The Ph.D.-12™ Phage Display Peptide Library Kit was obtained from New England BioLabs, Inc., Beverly, MA. This is a combinatorial peptide 12-mers fused to the minor coat protein (pIII) of M13 phage. The displayed peptide 12-mers are expressed at the N-terminus of pIII. The library consists of about 1.9 \(\times\) 10\(^{10}\) electroporated sequences, amplified once to yield about 20 copies of each sequence in 10 \(\mu\)l of the supplied phage.

Dodecapeptide Library Screening

Four biopannings were carried out according to the instruction of the manufacturer with some modifications. Briefly, 10 \(\mu\)g of biotinylated anti-NS3 mAb were coupled to 35 mm polystyrene petri dish (BD Falcon, San José, CA) coated with 40 \(\mu\)g of streptavidin. The dish was incubated overnight at 4°C and washed six-times with 50 mM Tris, 150 mM NaCl, pH 7.5 (TBS) containing 0.5% Tween-20 (TBS-T). In the first round of biopanning, 4 \(\times\) 10\(^{10}\) phages from the initial library were allowed to react with the dish bound IgG for 4 hr at 4°C under rocking condition. The unbound phages were removed by repetitive washes with TBS-T. The bound phages were then eluted from the dish with 400 \(\mu\)l of elution buffer (0.1 N HCl, pH adjusted to 2.2 with glycine, 1 mg/ml BSA). After neutralization with 75 \(\mu\)l of 1 M Tris-HCl pH 9.1, the eluted phages were then amplified by infecting 20 ml of a 1:100 dilution of an overnight culture of E. coli ER2537 (recA + strain cells), as recommended in the instruction manual. The culture was incubated for 4.5 hr at 37°C with vigorous shaking. The supernatants were obtained and precipitated with PEG as described previously [Scott and Smith, 1990].

The second, third, and fourth rounds of biopanning, 20% of the amplified phages from the preceding round was preincubated, overnight at 4°C, with the biotinylated anti-NS3 mAb at the final concentration of 10, 1, and 0.1 nM, respectively, before adding it to the 35-mm polystyrene petri dish coated with 10 \(\mu\)g of streptavidin. The procedure was then identical to the first round.

The phages from the fourth biopanning eluate were then cloned and amplified for DNA sequencing and immunoanalysis.

ELISA Using Supernatant Phages and Monoclonal Antibodies

Rows of ELISA plate wells were coated with 100 \(\mu\)l of either an anti-NS3 mAb or an irrelevant mAb, at the final concentration of 100 \(\mu\)g/ml, in 0.1 M NaHCO\(_3\) buffer (pH 8.6). The plates were first incubated overnight at 4°C and then blocked with 0.1 M NaHCO\(_3\) buffer (pH 8.6) containing 50 mg/ml of bovine serum albumin. After 2 hr of incubation at 4°C, the plates were washed six-times with TBS containing 0.5% Tween (TBS-T). Four fold serial dilutions of each phage clone were added to each well of the microtiter plate in a final volume of 100 \(\mu\)l of TBS-T, starting with 10\(^{12}\) virions in the first well of a row and ending with 2 \(\times\) 10\(^{7}\) virions in the 12th well. The plates were incubated for 2 hr at room temperature with agitation and then washed six-times with TBS-T, as above. The bound phages were detected in a sandwich assay using a horseradish peroxidase-conjugated
anti-M13 mAb at a 1:5,000 dilution (Amersham PharmaciaBiotech Inc., Piscataway, NJ). After 10 min of incubation with a commercial color kit (bioMérieux) containing ortho phenylene diamine and hydrogen peroxide, the reaction was stopped with 1.8 N H$_2$SO$_4$ and the plates were read at 492 nm with an ELISA plate reader. For each phage clone dilution, the results were expressed as the difference between the value obtained when using the tested anti-NS3 mAb and that when using the irrelevant mAb. The results were then confirmed by testing optimal dilutions of the immunoreactive clones in triplicate.

**ELISA Using Supernatant Phages and Human Sera**

Rows of ELISA plate wells were coated with 100 μl of either HCV positive sera or HCV negative sera, diluted 1:50, in 0.1 M NaHCO$_3$ buffer (pH 8.6). The following steps were identical to those described above for ELISA using supernatant phages and monoclonal antibodies.

**Peptide Synthesis on Nitrocellulose-Membrane**

The simultaneous synthesis of different peptide sequences was performed on a nitrocellulose membrane using 9-fluorenylmethoxycarbonyl amino acid chemistry [Frank and Döring, 1988]. Each peptide was generated in nanomolar quantities suitable for immunological detection. Antibody reactivity to membrane bound peptides was analyzed by an indirect colorimetric immunoassay, as described previously [Jolivet-Reynaud et al., 1998].

**Synthesis of Soluble Peptides**

Peptides corresponding to the selected regions were synthesized on an Applied Biosystems automatic synthesizer (model 431A) using fluorenylmethoxycarbonyl and t-buty1 protecting groups and trifluoroacetic acid (TFA) deprotection. Tetrabranching multiple peptides (MAP4) were synthesized according to Tam [1988].

The peptides were then purified by reverse-phase HPLC using an acetonitrile gradient containing 0.1% TFA as eluant. Composition of the purified peptides was confirmed by amino acid analysis and mass spectrometry.

**Inhibition of mAb Binding to Motif Phages by NS3 Peptides**

Five micrograms of peptide diluted in 50 μl of Tris-HCl 0.1 M pH 7.2 were incubated for 30 min at 37°C with mAb 3B1C4 coated on ELISA plate before adding 5 × 10$^{10}$ phages diluted in 50 μl of TBS-T. The subsequent steps were identical as described above for ELISA using supernatant phages and monochlonal antibodies.

**ELISA Using MAP4 Peptides and Human Sera**

Ninety-six well plates Maxisorb were coated with 100 μl/well of MAP4 peptide at the concentration of 10 μg/ml in 0.1 M carbonate buffer, pH 8.3. After 2 hr of incubation at 37°C, the plates were washed three-times with PBS containing 0.05% Tween-20 (PBS-T) and blocked for 1 hr at 37°C with PBS containing 10% goat serum. Following a second wash with PBS-T, 100 μl of human sera diluted in PBS-T containing 10% goat serum were added and incubated for 2 hr at 37°C. After a new wash with PBS-T, the secondary antibody, peroxidase-conjugated goat anti-human IgG (H + L) (Jackson ImmunoResearch Laboratories), was then added at a 1:5,000 dilution in PBS-T-goat serum. The plates were incubated for 1 hr at 37°C and then washed once more with PBS-T. After 10 min of incubation with a commercial color kit (bioMérieux) containing ortho phenylene diamine and hydrogen peroxide, the reaction was stopped with 1.8 N H$_2$SO$_4$ and the plates were read at 492 nm with an ELISA plate reader. The values are expressed as the mean OD of triplicate. Statistical analysis of the specificity of recognition of the MAP4-peptides by HCV + positive sera compared to control sera was determined by Student’s t-test. P-values of less than 0.05 were considered significant.

**Sequence Analysis**

The amino acid sequences of peptides were compared to the NS3 recombinant protein sequence by use of the Mac Vector, ver. 4.5 software (Kodak). Basically, the regions with the highest similarity were detected with the Clustal W program, which searches tentatively for best local identities.

**Molecular Display**

The three-dimensional structure of the NS3 helicase molecule was obtained from the Protein Data Bank (PDB code: 1HEI). Visualization of the molecule was carried out with the help of WebLab ViewerLite 3.2 (Molecular Simulation, Inc.).

**RESULTS**

**Characterization of Anti-NS3 Monoclonal Antibodies**

Fifteen hybridomas secreting mAbs were identified for their recognition of both HCV NS3(272) recombinant proteins, as well as of both NS3(93) truncated recombinant proteins. Among these different mAbs, mAb 9 (3B1C4) competed with all other anti-NS3 mAbs and, more particularly, with mAb 2 for binding to NS3(272). Furthermore, the respective mAb 9 and mAb 2 binding were also inhibited strongly by a pool of NS3 positive human sera (Table I) suggesting that mAb 9 (3B1C4) and mAb 2 (12A1H2) should be directed against an immunodominant epitope of NS3 also recognized by the human immune system. However, to further analyze this epitope, we selected 3B1C4 since its affinity constant $K_a = k_a/k_d$ (M$^{-1}$) for NS3 was higher than the one of 12A1H2 (8.3 × 10$^7$ and 1.5 × 10$^7$, respectively).

In a preliminary approach, 8 and 15 mers overlapping peptides offset by one and spanning the NS3-93
sequence (residues 1372–1464) were synthesized onto nitrocellulose membrane using the Spots technology [Frank and Döring, 1988], and tested for their immuno-reactivity with 3B1C4. However, the different membrane bound peptides were not recognized by 3B1C4 indicating that the epitope was likely conformational.

Selection of Phage Displayed Peptides Mimicking the 3B1C4 Epitope

Random peptide libraries are effective tools to determine both linear and conformational epitopes. For this reason, a library of dodecapeptides displayed on phage was screened with 3B1C4 as an attempt to identify determinants recognized by this mAb. After four rounds of biopanning, 35 phage clones were selected at random and their DNA were sequenced. As shown in Table II, the amino acid sequences of the inserts evidenced the selection of 26 different motifs which were tested by ELISA for their immunoreactivity with mAb 3B1C4. Seventeen clones gave a positive signal but three of them (clones 6, 16, and 17) appeared to be more significantly recognized by 3B1C4. Interestingly, clones 6 and 16 were also recognized by the different anti-NS3 monoclonal antibodies which competed with 3B1C4 for NS3-272 binding whereas the signal induced by clone 17 was weak (Fig. 1).

Immuno-reactivity of the Selected Phage Clones With Anti-NS3 Human Sera

In a preliminary experiment, the phage clones 6, 16, and 17 were tested for their immunoreactivity with a pool of 5 NS3 positive human sera and a pool of sera from 5 healthy individuals, both diluted 1:50. Clones 6 and 16 were specifically recognized by HCV + sera, whereas the respective signals of clone 17 with either HCV positive sera or negative sera were not significantly

| TABLE I. Competition of 3B1C4 With the Anti NS3 mAbs and Human Sera |
|-------------------------------|-----------------|-----------------|-----------------|
| First mAb                      | Second biotinylated mAb |
|--------------------------------|-------------------|-----------------|
| mAb 9 (3B1C4)                  | 1                | 2               | 15              |
| Human sera                     | 45               | 75              | 3               |

ELISA competition experiments were performed as described in “Materials and Methods.” Results are expressed as inhibition percentage of the second antibody binding to the recombinant NS3(272)-His by either mAb 9 or HCV positive human sera compared to the controls measured under identical conditions but in the absence of either mAb 9 or human sera, respectively. Strong inhibitions are highlighted in bold.

| TABLE II. Frequency and Immunoreactivity of Phage Displayed Peptides Selected With 3B1C4 |
|---------------------------------------------|-----------------|-----------------|
| Motif number                               | Clone sequence  | Clone frequency | Clone reactivity |
| 1   | H  | K  | M  | H  | S  | H  | P  | R  | L  | T  | S  | P  |   | 6/35                      | 0.128 |
| 2   | F  | H  | K  | H  | S  | P  | R  | S  | P  | I  | F  | I  | 1/35                   | 0.317 |
| 3   | W  | H  | K  | A  | V  | P  | R  | W  | L  | A  | S  | P  | 3/35                   | 0.543 |
| 4   | F  | H  | K  | H  | K  | S  | P  | A  | L  | S  | P  | V  | 2/35                   | 0.479 |
| 5   | F  | H  | G  | H  | L  | K  | K  | P  | H  | W  | R  | N  | 2/35                   | 0.154 |
| 6   | W  | H  | R  | H  | W  | P  | S  | H  | P  | T  | Q  | K  | 1/35                   | 1.615 |
| 7   | G  | L  | L  | H  | K  | H  | H  | R  | S  | P  | Y  | 1/35                   | 0.456 |
| 8   | D  | L  | N  | Y  | F  | T  | L  | S  | K  | R  | E  | 1/35                   | 0.177 |
| 9   | F  | H  | K  | H  | R  | I  | S  | P  | S  | P  | S  | T  | 1/35                   | 0.252 |
| 10  | A  | T  | A  | K  | H  | L  | Y  | W  | W  | R  | N  | Q  | 1/35                   | 0.656 |
| 11  | W  | H  | K  | G  | N  | N  | V  | A  | W  | T  | K  | R  | 1/35                   | 0.430 |
| 12  | W  | H  | S  | H  | M  | K  | T  | R  | T  | W  | Q  | P  | 1/35                   | 0.543 |
| 13  | F  | H  | S  | P  | K  | N  | H  | H  | Y  | Y  | R  | 1/35                   | 0.507 |
| 14  | H  | H  | K  | H  | S  | N  | R  | S  | P  | I  | F  | S  | 1/35                   | 0.224 |
| 15  | F  | H  | H  | K  | H  | S  | P  | R  | L  | F  | P  | 1/35                   | 0.268 |
| 16  | A  | H  | K  | W  | Y  | S  | Q  | W  | L  | P  | H  | R  | 1/35                   | 1.461 |
| 17  | W  | H  | H  | R  | H  | Q  | P  | A  | P  | G  | G  | R  | 1/35                   | 1.021 |
| 18  | A  | K  | L  | H  | W  | H  | K  | H  | P  | L  | T  | 1/35                   | <0.1 |
| 19  | F  | H  | K  | H  | N  | Y  | K  | S  | P  | P  | I  | 1/35                   | <0.1 |
| 20  | W  | H  | K  | V  | P  | R  | S  | D  | R  | M  | P  | P  | 1/35                   | <0.1 |
| 21  | F  | H  | K  | Y  | S  | P  | P  | Q  | K  | P  | V  | T  | 1/35                   | <0.1 |
| 22  | W  | P  | H  | F  | H  | R  | P  | S  | P  | H  | R  | E  | L  | 1/35                   | <0.1 |
| 23  | H  | L  | Y  | H  | K  | N  | R  | N  | H  | I  | A  | Y  | 1/35                   | <0.1 |
| 24  | I  | Q  | R  | H  | H  | K  | P  | L  | R  | L  | R  | V  | 1/35                   | <0.1 |
| 26  | F  | H  | K  | H  | D  | R  | G  | R  | L  | S  | P  | P  | 1/35                   | <0.1 |
| 25  | S  | W  | R  | S  | R  | Q  | L  | P  | E  | T  | G  | E  | 1/35                   | <0.1 |

The immunoreactivity of phage clones was determined by ELISA, as described in “Materials and Methods.” The results are expressed as the mean optical density (OD) obtained with triplicate of phage clones tested, at the final dilution of 10^11 phages/ml with 3B1C4 coated on the solid phase minus the mean OD of triplicate of the same phage dilutions tested with an irrelevant mAb.
different. Thus, the clones 6 and 16 were tested with 12 sera of healthy individuals and 12 HCV positive sera in which anti-NS3 antibodies had been evidenced previously by RIBA. For each clone, a cut-off of positivity (mean of the 12 values obtained with HCV$^+$ sera + 2 standard deviations) allowed to characterize 12 and 11 positive responses against clone 6 and 16, respectively (Fig. 2).

**3B1C4 Epitope Localization**

The selected mimotopes mimic the binding characteristics of the natural epitope. In the case of noncontinuous epitope, there may be little or no homology with the antigen sequence [Yayon et al., 1993; Ludmerer et al., 1996]. However when compared to the sequence of NS3 helicase domain within the region 1372–1464 corresponding to the recombinant protein NS3(93) which was well recognized by the anti-NS3 mAbs, WRRHWPSHPTQK (clone 6) and AHKWYSQWLPHR

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**Fig. 1.** Recognition of phage clones 6, 16, and 17 by the anti-nonstructural 3 (NS3) protein mAbs. The immunoreactivities of the phage clones 6, 16, and 17 (histograms in white, grey, and black, respectively) were tested as described in “Materials and Methods” at the final dilution of $10^{11}$ phages/ml with each of the different anti-NS3 mAbs coated on the solid phase. The results are expressed as in Table II.

**Fig. 2.** Recognition of the phage clones 6 and 16 by human sera. The immunoreactivities of the phage clones were tested as described in “Materials and Methods” at the final concentration of $3.3 \times 10^{11}$ phages/ml with sera diluted to 1/50. Histograms in white correspond to sera from healthy individuals and histograms in black to hepatitis C virus (HCV) NS3$^+$ sera. The horizontal line indicates the cut-off of specific recognition.
(clone 16) shared some homologies within the NS3 sequences 1389–1398 and 1376–1381, respectively (Fig. 3). Indeed, three amino acids of clone 6 were identical to R1389, H1390, K1398 and two amino acids were similar to S1396, K1397 whereas two amino acids of clone 16 were identical to Y1376, P1381, and two amino acids were similar to K1378 and I1380. When tested by ELISA, in direct binding, synthetic peptides I18G and G19L corresponding to NS3 sequences 1392–1411 and 1371–1391, respectively, were not recognized by either 3B1C4 or a pool of anti-NS3 positive sera. However, inhibition experiments showed that the peptide I18G inhibited 73.4% and 57.4% of the 3B1C4 binding to clone 6 and 16, respectively (Fig. 4). On the contrary, peptide G19L had no significant effect on 3B1C4 binding to both clones as well as K18F another peptide located to the HCV sequence 1191–1210. These results confirm the conformational characteristics of the 3B1C4 epitope which cannot be reproduced by short linear peptides of the NS3 sequence but be mimicked by clone 6 and 16 which may contain residues identical or similar to noncontinuous residues of NS3 present throughout the sequence.

**Molecular Modeling of the 3B1C4 Epitope**

As an attempt to visualize the 3B1C4 epitope, we identified, on the three-dimensional structure of NS3 helicase domain [Yao et al., 1997], the amino acids that were shown to be identical or similar to clone 6 and 16 amino acids (Fig. 3). S1396, K1397, and K1398 (residues 10, 11, and 12 of clone 6) were localized outside of the dimer domain interaction in a loop very accessible to antibody recognition (Fig. 5). However, amino acids R1389, H1390 identical to clone 6 amino acids 3 and 4, respectively, were located on the other side of the molecule indicating that these identities were not relevant. Y1376 and K1378, which are similar to clone 16 amino acids 5 and 7, respectively, appeared to be adjacent to the linear stretch of amino acids 1396–1398 whereas I1380 and P1381 (similar and identical to residues 9 and 10 of clone 16, respectively) were buried in the molecule. This data suggests that the 3B1C4 epitope could be composed of at least Y1376, K1378, S1396, K1397, and K1398. However, according to the respective abilities of I18G and G19L to inhibit the 3B1C4 binding to clone 6 and 16, the interaction between 3B1C4 and NS3 should involve mainly the amino acids (S1396, K1397, and K1398) contained in I18G.

**Immunoreactivity of Mimotopes 6 and 16 as Synthetic Peptides**

The NS3 mimotopes 6 and 16 were reproduced as branched synthetic peptides (MAP4-6 and MAP4-16, respectively) according to Lu et al. [1991] and tested first with 57 sera from healthy individuals. It allowed the determination of a cut-off of specific recognition for each peptide (mean of the values obtained with the 57 HCV sera + 2 standard deviations). When tested with 49 anti-NS3 positive human sera, MAP4-6 was recognized specifically by 19 sera and MAP4-16 by 22 sera (Fig. 6)
Fig. 6. Recognition of MAP4-6 and MAP4-16 by anti-NS3 positive human sera. The peptides were tested with sera diluted to 1/50. Histograms in black correspond to values obtained with anti-NS3 positive sera, histograms in black to values obtained with control sera.

a: Response with MAP4-6. The horizontal line represents the cut-off of specific recognition calculated as indicated in the text: $(0.119 + (2 \times 0.077)) = 0.272$.

b: Response with MAP4-16. The horizontal line represents the cut-off of specific recognition calculated as indicated in the text: $(0.144 + (2 \times 0.073)) = 0.289$.
and the combination of these two peptides detected a positive response in 27 out of the 49 tested sera. In addition, statistical analysis showed that the 49 HCV positive sera values were significantly different from the 57 control values for both MAP4-6 and MAP4-16 recognition ($P = 1.7 \times 10^{-5}$ and $3.3 \times 10^{-7}$, respectively).

When tested with an anti-HCV seroconversion panel, MAP4-6 was also recognized by 3 out of 18 seroconversion sera and MAP4-16 by 7 out of 18 (Table III). The combination of the two peptides allowed the detection of eight positive sera.

**DISCUSSION**

By screening a dodecapeptide library displayed on phage with the anti-NS3 mAb 3B1C4, we have selected two motifs recognized by most of the anti-NS3 mAbs competing with 3B1C4 and by human anti-NS3 antibodies from HCV infected patients. The similarity of these motifs with NS3, as well as the inhibition of the 3B1C4 binding to these motifs by NS3 synthetic peptides, allowed us to localize the minimal sequence of the epitope within the residues 1376–1398. Mondelli et al. [1994], first characterized a major conformational B-epitope on NS3 defined by the human monoclonal antibody CM3.B6. This epitope was localized in a subregion covered by residues 1378–1443. Interestingly, it was shown that this human mAb identified a unique B-cell epitope which was recognized predominantly by sera from viremic HCV-carriers. Zhang et al. [2000] characterized recently a murine mAb (ZX10) recognizing the NTPase/helicase domain of the HCV. This mAb recognized a discontinuous epitope of which the linear sequence GEIPFYGKAIP at residues 1371–1382 constituted one part. In our data, clone 16 shared some similarities in the region 1376–1382, suggesting that 3B1C4 could recognize the same epitope than the ZX10 mAb. However, the binding of 3B1C4 to clone 16 as well as to clone 6 was inhibited by the synthetic peptide P18G (residues 1391–1411) but not by the peptide G19L (residues 1371–1391). ZX10 did not bind the synthetic peptide YGKAIPL. Therefore, the 3B1C4 epitope should be close to but yet different from the ZX10 one. In contrast, 3B1C4 could be similar to the human mAb CM3.B6 located in the same region [Mondelli et al., 1994] since the phage clones selected by 3B1C4 appeared to be recognized by NS3 positive human sera.

Using another approach, Pereboeva et al. [2000] created libraries expressing HCV protein fragments on the fUSE2 phage surface and identified an antigenic determinant in the same region of NS3 (amino acids 1383–1415) by panning with a mixture of sera from five HCV-positive individuals. However, their attempts to identify epitopes in NS3 by screening a phage displayed library of random 15-mer peptides with either whole human sera or affinity purified anti-NS3 antibodies had failed [Pereboeva et al., 1998].

In our approach, the use of an anti-NS3 mAb competing strongly with HCV + human sera for the screening of the dodecapeptide library allowed to successfully obtain clones mimicking an immunodominant NS3 epitope. Both inhibition experiments with synthetic peptides covering the NS3 (93) sequence and amino acids similarities between clone 6 and NS3 allowed to localize part of the epitope at residues S1396, K1397, and K1398 and to visualize them on the three-dimensional structure of the NS3 helicase domain [Yao et al., 1997] on a very accessible loop outside of the dimerization domain. However, Y1376 and K1378, which were similar to amino acids 5 and 7 of clone 16, were located beside residues 1396–1398 on the three-dimensional structure, suggesting they could also interact to a limited extent with 3B1C4. According to Yao et al. [1997], K1386, R1389 and the cluster K (1397, 1398, and 1399) are candidates for favorable electrostatic RNA helicase interactions. Thus, the ability of the 3B1C4 antibody to interfere with the enzymatic functions of NS3 has to be investigated.

Minenkova et al. [2001] reported the identification of a wide collection of efficient HCV-specific ligands and the development of a novel type of diagnostic kit referred to as ADAM (antibody detection by antigen mimics) for the detection of anti-HCV antibodies in the serum. Basically, these HCV-specific ligands were obtained by applying different selection strategies for the screening of phage libraries using sera from HCV-infected patients and non-infected individuals. These selected ligands were later improved by in vitro strategies [Urbanelli et al., 2000; Zhu et al., 2000]. Among the different immunoreactive ligands, the four NS3 mimotopes mimicked a different NS3 major epitope located at the 1264–1274 residues in the helicase domain.

**TABLE III. Recognition of MAP4-6 and MAP4-16 by an Anti-HCV Seroconversion Panel**

<table>
<thead>
<tr>
<th>Sera</th>
<th>RIBA NS3a</th>
<th>MAP4-6</th>
<th>MAP4-16</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC1</td>
<td>+/−</td>
<td>0.158 (+)h</td>
<td>0.301 (+)</td>
</tr>
<tr>
<td>SC2</td>
<td>+/−</td>
<td>0.173 (+)h</td>
<td>0.219 (+)</td>
</tr>
<tr>
<td>SC3</td>
<td>+/−</td>
<td>0.206 (+)h</td>
<td>0.323 (+)</td>
</tr>
<tr>
<td>SC4</td>
<td>4+</td>
<td>0.172 (+)</td>
<td>0.213 (+)</td>
</tr>
<tr>
<td>SC5</td>
<td>4+</td>
<td>0.174 (+)</td>
<td>0.213 (+)</td>
</tr>
<tr>
<td>SC6</td>
<td>4+</td>
<td>0.184 (+)</td>
<td>0.225 (+)</td>
</tr>
<tr>
<td>SC7</td>
<td>4+</td>
<td>0.237 (+)</td>
<td>0.334 (+)</td>
</tr>
<tr>
<td>SC8</td>
<td>4+</td>
<td>0.264 (+)</td>
<td>0.390 (+)</td>
</tr>
<tr>
<td>SC9</td>
<td>4+</td>
<td>0.303 (+)</td>
<td>0.433 (+)</td>
</tr>
<tr>
<td>SC10</td>
<td>3+</td>
<td>0.253 (+)</td>
<td>0.355 (+)</td>
</tr>
<tr>
<td>SC11</td>
<td>4+</td>
<td>0.374 (+)</td>
<td>0.400 (+)</td>
</tr>
<tr>
<td>SC12</td>
<td>+/−</td>
<td>0.243 (+)</td>
<td>0.183 (+)</td>
</tr>
<tr>
<td>SC13</td>
<td>+/−</td>
<td>0.251 (+)</td>
<td>0.175 (+)</td>
</tr>
<tr>
<td>SC14</td>
<td>+/−</td>
<td>0.431 (+)</td>
<td>0.176 (+)</td>
</tr>
<tr>
<td>SC15</td>
<td>+/−</td>
<td>0.234 (+)</td>
<td>0.209 (+)</td>
</tr>
<tr>
<td>SC16</td>
<td>1+</td>
<td>0.209 (+)</td>
<td>0.280 (+)</td>
</tr>
<tr>
<td>SC17</td>
<td>2+</td>
<td>0.209 (+)</td>
<td>0.229 (+)</td>
</tr>
<tr>
<td>SC18</td>
<td>2+</td>
<td>0.217 (+)</td>
<td>0.227 (+)</td>
</tr>
</tbody>
</table>

The peptides were tested by ELISA as described in “Materials and Methods” with sera diluted to 1/50.

*aPresence of NS3 antibodies evaluated by RIBA according to the indication of the manufacturer.

hThe cut-off of MAP4-6 recognition calculated as indicated in the text was: (0.119 + (2 x 0.0777)) = 0.272. The values under and above the cut-off are indicated as (−) and (+), respectively.

The cut-off of MAP4-16 recognition calculated as indicated in the text was: (0.144 + (2 x 0.0753)) = 0.289. The values under and above the cut-off are indicated as (−) and (+), respectively.
However, MAP4-6 and MAP4-16 mimicked also a major NS3 epitope since they were detected by 19 and 22, respectively, out of 49 anti-NS3 positive sera. Interestingly, even if the two mimotopes were selected with the same mAb, the mimicry of the corresponding epitope was not identical since the cumulative recognition of these 2 peptides allowed to increase the detection of anti-NS3 antibodies to 27 out of the 49 tested sera. The RIBA analysis detected an anti-NS3 response in sera from patient with seroconversion. Although corresponding to only one NS3 epitope, MAP4-6 and MAP4-16 were also recognized by 3 and 7 sera, respectively, out the 18 seroconversion. We also noted that positivities were seen even in sera in which anti-NS3 antibodies were only weakly detected by RIBA. These results suggest that the antibodies directed against the epitope mimicked by MAP4-6 and MAP4-16 appear early in the course of infection.

Several recombinant proteins or synthetic peptides such as C100-3, c22, e1, e2, C33C, and ns5 reproducing part HCV antigens (NS4, core, E1, E2, NS3, and NS5, respectively) are used currently in commercial diagnostic kits for the detection of anti-HCV antibodies in patients [Chaudhary and Jacobsen, 1994; Ou-Yang et al., 1999] and a positive diagnosis requires a positive response against at least two antigens. The detection of anti-HCV antibodies with a sensitivity of 92.75% and a specificity of 100% by ADAM-HCV EIA needs a mix of 15 mimotopes corresponding to several immunodominant epitopes of HCV structural and non structural proteins [Minenkova et al., 2001]. However, the efficiency of this HCV assay proves that disease specific mimotopes can be used as antigen-coated phase instead of recombinant proteins. The data, presented above, confirm this approach since the peptides MAP4-6 and MAP4-16, as mimotopes corresponding to only one NS3 epitope, are already valuable tools for the detection of HCV antibodies.

REFERENCES


Chaudhary RK, Jacobsen H. 1994. Performance of third-generation epitope mimotopes can be used as antigen-coated phase instead of recombinant proteins. The data, presented above, confirm this approach since the peptides MAP4-6 and MAP4-16, as mimotopes corresponding to only one NS3 epitope, are already valuable tools for the detection of HCV antibodies.


