Journal of Peptide Science
J. Peptide Sci. 2006; **12**: 267–278
Published online 23 September 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.719

JOURNAL OF PEPTIDE SCIENCE

Antigenicity of chimeric and cyclic synthetic peptides based on nonstructural proteins of GBV-C/HGV

T. PÉREZ,[a] G. ERCILLA,[b] W. C. CHAN[c] and I. HARO[a]*

[a] Department of Peptide and Protein Chemistry, IIQAB-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain
[b] Immunology Service, ICII-IDIBAPS, Hospital Clínic, Barcelona, Spain
[c] School of Pharmacy, Centre for Biomolecular Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

Received 25 April 2005; *Revised 17 June 2005*; *Accepted 11 July 2005*

Abstract: In this work, new putative epitopes located in nonstructural proteins of GBV-C/HGV were synthesized using solid-phase chemistry for their use in immunoassays. The antigens were obtained in linear, chimeric and cyclic forms with the main aim of improving the sensitivity of the enzyme immunoassays. Our results showed, on one hand, that the combination of different antigens seems to be necessary to ensure good sensitivity and more specificity and, on the other hand, that cyclic compounds show higher ability to recognize anti-GBV-C/HGV antibodies than its parent peptide. Furthermore, CD and FTIR have been used in conjunction to characterize the conformational changes therein with synthetic constructs that could explain their different antigenicity. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: GBV-C/HGV; diagnosis; solid-phase peptide synthesis; chimeric peptides; cyclic peptides; ELISA; circular dichroism; Fourier transformed infrared spectroscopy

INTRODUCTION

GB virus C (GBV-C), also called hepatitis G virus (HGV), is a single-strand RNA virus belonging to the Flaviviridae family. The GB virus C/Hepatitis G virus (GBV-C/HGV) has spread worldwide and virus infections are extremely common, having been found among healthy blood donors and also at a high rate in individuals with parenteral risk factors, suggesting that this virus is transmitted parenterally by transfusion of blood components, intravenous drug use and vertically/perinatally. Coinfections with hepatitis B and hepatitis C are common, but controversial data exist concerning their potential to cause hepatitis in humans [1–4]. However, recent reports raised interest in this apparently nonpathogenic virus because of a potential beneficial effect of the GBV-C/HGV infection that is associated with prolonged survival in HIV-positive people [5]. The appearance of anti-E2 antibodies is usually taken as an indication of recovery of GBV-C/HGV infection [6], and in spite of people rarely having concurrent GBV-C/HGV anti-E2 antibodies and GBV-C/HGV viremia [7,8], more data on the development of antibodies are needed to understand the effect of previous exposure to GBV-C/HGV into the progression of HIV disease.

GBV-C/HGV viremia could be detected by a reverse transcription-polymerase chain reaction (RT-PCR) [9]. Currently, an assay that detects antibodies to the GBV-C/HGV envelope E2 protein has also been developed and is commercially available [10]. However, the use of recombinant proteins such as antigens can be sometimes disappointing, mainly because they may produce false-positive results as previously reported [11,12]. In contrast, synthetic peptides provide a uniform, chemically well-defined antigen, thus reducing inter- and intra-assay variation. In this sense, epitopes other than those located on the E2 structural protein would be able to elicit a humoral immune response. In recent years, synthetic peptides that mimic specific epitopes of infectious agents have been used in diagnostic systems for various diseases [13–16]. In the context of using synthetic peptides or peptide derivatives for the diagnosis of GBV-C/HGV infections, Toniutto *et al.* [16] studied putative B-cell epitopes in the nonstructural regions 4 (NS4) and 5 (NS5) of the GBV-C/HGV polyprotein. In our group, new epitopes located in structural (E2) and NS3 proteins of GBV-C/HGV were also identified and synthesized for their use in immunoassays [17]. However, short peptides representing topographic B-cell epitopes are generally poorly antigenic. Although two

Abbreviations: Abs, absorbance; CD, circular dichroism; Boc, *tert*-butoxycarbonyl; DIEA, *N*,*N*-diisopropylethylamine; DIPCDI, *N*,*N*-diisopropylcarbodiimide; DMF, dimethylformamide; ivDde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methybutyl; Dmab, 4-(*N*-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl)amino)benzyl; EDT, ethanedithiol; ELISA, enzyme-linked immunosorbent assay; Fmoc, fluoren-9-ylmethoxycarbonyl; FTIR, Fourier transform infrared spectroscopy; GBV-C/HGV, GB virus C/Hepatitis G virus; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; HMPA, 4-hydroxymethylphenoxy-acetic acid; HOBt, *N*-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption-ionization time-of-flight mass spectrometry; MeCN, acetonitrile; NS, nonstructural; PBS, phosphate buffered saline; PBS-T, phosphate buffered saline and Tween; PyBOP, Benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; SPPS, solid-phase peptide synthesis; TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TFE, trifluoroethanol

*Correspondence to: I. Haro, Department of Peptide and Protein Chemistry, IIQAB-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain; e-mail: ihvqpp@iiqab.csic.es

Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

or more antigens can be used in the assays, the sensitivity and specificity can be affected by the competition for binding on the solid phase and for changes in the spatial distribution of antigenic determinants of bound peptides. There is a tendency toward using chimeric peptides to avoid those problems and to improve the sensitivity and specificity of the assays [13,18,19].

Another problem is that peptides are conformationally heterogeneous in aqueous solution, often differing from the structures their cognate sequences adopt in the native protein [20]. As a result, the affinity of peptides for antibodies is reduced. A way to overcome this problem is by constraining the peptide to conformations that favor antibody binding by means of macrocyclization [21,22]. Homodetic cyclopeptides can be conveniently synthesized by solid-phase synthesis by anchoring a trifunctional amino acid to the resin through its side chain. The combination of this strategy with an orthogonal three-dimensional protection scheme, such as Fmoc/*t*Bu/Dmab solid-phase peptide synthesis (SPPS), results in a powerful cyclization methodology [23,24].

In the present work, new putative epitopes located in two nonstructural proteins of GBV-C/HGV were identified by computer-aided prediction of antigenicity and were chemically synthesized using solid-phase peptide chemistry. The corresponding synthetic peptides NS4a(27–43), NS4b(8–22) and NS5a(112–126) were used as antigens in an enzyme-linked immunosorbent assay (ELISA).

With the purpose of raising an adequate antigenic response, the colinear linking of NS4b(8–22) and NS5a(112–126) antigens was carried out. Thus, two chimeric peptides (Qm1, Qm2) with two different epitope orientations and a triglycyl spacer between the epitopes were also synthesized. In addition, two cyclic versions of the NS4a(27–43) peptide sequence containing either a head-to-tail (cyc-HT) or a side-chain-to-tail (cyc-CT) ring were prepared. All these synthetic constructs were evaluated by ELISA to establish whether the epitopes in chimeric or cyclic peptides are more efficiently recognized by the specific antibodies compared to the monomeric linear sequences.

In this paper, we also describe our findings concerning the comparative conformational analysis, by circular dichroism (CD) and Fourier transform infrared spectroscopy (FTIR), of the parent linear sequences and the different peptide constructs. These assays were carried out with the aim of establishing a relationship between conformation and antigenicity.

MATERIALS AND METHODS

Peptide Synthesis

Monomeric and chimeric GBV-C/HGV nonstructural linear peptides were synthesized manually in a NovaSyn® TGA resin (0.23 mmol g^{-1}) as *C*-terminal acids by solid-phase synthesis. The resin and the protected amino acids were obtained from Novabiochem Ltd (Switzerland).

Peptide synthesis was performed using standard Fmoc/*t*Bu solid-phase chemistry. The resin (1 g) was acylated with the symmetrical anhydride of Fmoc-Asp-ODmab. This special protected amino acid was used with the purpose of using the same resin-bound peptide intermediate, in a divergent manner, for the synthesis of both linear and cyclic peptides. For the sequence elongation, couplings were carried out by carboxyl activation using *N*,*N*-diisopropylcarbodiimide (DIPCDI) and *N*-hydroxybenzotriazole (HOBt). For difficult couplings, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and *N*,*N*-diisopropylethylamine (DIEA) were used as activating mixtures. The efficiency of these reactions was evaluated by the ninhydrin colorimetric reaction. Repeated couplings were carried out when a positive ninhydrin test was observed.

After completion of the peptide assembly, the resin was treated with 3% hydrazine in dimethylformamide (DMF) to remove the Dmab-protecting group. Then, peptides were deprotected and simultaneously cleaved from the resin with a mixture of TFA/EDT/H_2O (95/2.5/2.5), isolated by precipitation with cold diethyl ether, centrifuged and the residue material dissolved in distilled water and lyophilized. Crude peptides were purified by preparative high-performance liquid chromatography (HPLC) on a Shimadzu chromatograph equipped with a C18-silica column. The samples were eluted with a linear gradient from 90% A/10% B to 65% A to 35% B in 60 min (A: 0.05% TFA in H_2O; B: 0.05% TFA in acetonitrile (MeCN)) at a flow rate of 2 ml min^{-1} and the effluent monitored at 215 nm. Purified peptides were successfully characterized by analytical RP-HPLC, qualitative and quantitative amino acid analysis (Beckman 6300 System Gold) and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry MALDI-TOF MS (Voyager-DE RP, Biosystems).

The cyclic peptides were synthesized on a NovaSyn® TG Sieber resin (1 g, 0.16 mmol g^{-1}) following the procedures outlined in Figure 3. The resin was firstly deprotected with piperidine/DMF and acylated with activated Fmoc-β-Ala-OH; secondly, it was deprotected in the same basic conditions and acylated with activated 4-hydroxymethylphenoxyacetic acid (HMPA) and finally it was esterified with preformed Fmoc-Asp-ODmab anhydride via its side chain. Peptide synthesis was then continued using standard Fmoc/*t*Bu strategy.

To obtain cyc-CT, the side chain of ^{32}Lys was protected with 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methybutyl (ivDde), while the *N*-terminal Thr residue was protected with *tert*-butoxycarbonyl (Boc). Prior to on-resin cyclization, N^{ε}-ivDde and C^{α}-Dmab groups were removed with 3% hydrazine in DMF followed by treatment with 20% water in DMF.

To obtain cyc-HT standard, protected amino acids were used. Following solid-phase peptide assembly, the *N*-terminal Fmoc group was removed with 20% piperidine in DMF, and the removal of C^{α}-Dmab group was carried out under the same conditions as mentioned above for cyc-CT.

The regio-selectively deprotected peptide-resins were washed with 1% DIEA-DMF followed by 1% HOBt-DMF. The on-resin macrocyclization reactions were carried out with 1.5 eq of benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) at ambient temperature for

Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

5 days, followed by acidolytic cleavage using a mixture of TFA/EDT/H_2O (95/2.5/2.5) to afford the crude peptides. The cyclic constructs were isolated by precipitation with cold diethyl ether; the residue material was dissolved in 10% aqueous acetic acid and lyophilized. The crude peptides were purified by reversed-phase HPLC and identified by mass spectrometry as previously described (Table 1).

Serum Specimens

Two panels of serum samples were studied. The first group consisted of 100 sera from hemodialyzed patients from the Hospital Clínic of Barcelona. The second group consisted of 100 sera from chronic hepatitis patients from the same hospital. Thirty-five samples of the hemodialyzed and the chronic hepatitis panels were tested for the presence of anti-HGV E2 protein (Roche Diagnostics + GmbH). To determine the cutoff (CO) value, 80 control sera from volunteer blood donors were studied and used as negative controls.

Noncompetitive ELISA

Peptides were coupled to assay plates (Costar Corp. DNA-Bind™ N-oxysuccinimide surface) at 0.5 μg per well. Coupling was performed overnight at room temperature at pH 9.6 (0.05 M carbonate/bicarbonate buffer). After incubation, the plates were washed three times with phosphate buffered saline (PBS). The remaining free binding sites were blocked with 3% skimmed milk powder in phosphate buffered saline and Tween (PBS-T) (PBS-T-milk) for 1 h. Sera samples were diluted 50-fold in PBS-T-milk. 100 μl of this solution was added to each well and the plates were incubated for 2 h at room temperature. After washing (three times with PBS-T), 100 μl of antihuman IgG conjugated to peroxidase (Dako P0214; Glostrup, Denmark) diluted 1/1000 in PBS-T-milk was added to the wells and incubated for 1 h at room temperature. After incubation, the plates were washed three times with PBS-T. Bound antibodies were detected with *ortho*-phenylendiamine dihydrochloryde (OPD, Sigma) and 30% H_2O_2 as substrate. After an incubation time of 30 min at room temperature, the reaction was stopped by adding 2M H_2SO_4 (50 μl per well) and absorbance values were measured at 492 nm.

Each plate contained control wells that included all reagents except the serum sample in order to estimate the background reading and control wells that included all reagents except the peptide to estimate nonspecific reactions between milk proteins and positive sera. All assays included positive and negative controls. Moreover, each plate contained control wells coated with an irrelevant peptide (a 19-mer peptide sequence of filaggrin protein) in order to control the specificity of the peptide–serum interactions. Samples were considered positive when the absorbance was equal to or higher than the CO. The CO value was calculated for each panel from the negative control sera (average plus two standard deviations).

Inhibition ELISA

In order to confirm the specificity of the peptide–antibody interactions, as well as to estimate the cross-reactivity between the sera and monomeric and chimeric peptides, competitive ELISA assays were carried out.

NS4b(8–22), NS5a(112–126), Qm1 and Qm2 either those in solution or those covalently bound to the ELISA plate were used. Inhibition enzyme immunoassays were carried out following the procedures described above, except the step of serum incubation. A highly positive control serum was added to each well containing 50,100 and 200 μg ml^{-1} of inhibiting peptide. The main inhibition (%) was calculated by comparing absorbance values in the presence and in the absence of an inhibitor.

Statistical Analysis

The reactivities of each peptide construct with the two panels of sera and the reactivities of the peptides obtained in the different chemistries used to link the peptides on the solid surface were compared using the *t*-test analysis. Probability values less than 0.05 were considered statistically significant.

Table 1 Primary sequences and peptide characterization by RP-HPLC and mass spectrometry of synthetic peptides used in this study

			MALDI-TOF-MS	
Name	Sequence	RP-HPLC[c]	Theory (Da)	Experimental (Da)
NS4a(27–43)	TDWDVKGGGSPLYRHGD	$k' = 2.88$[b]	1859.97	1859.90
NS4b(8–22)	GESAPSDAKTVTDAVD	$k' = 2.50$[a]	1562.61	1562.65
NS5a(112–126)	GTSGWAEVVVTPTHVD	$k' = 2.20$[a]	1654.80	1654.83
Qm1	NS5a(112–126)-GGG-NS4b(8–22)	$k' = 3.57$[a]	3371.54	3371.10
Qm2	NS4b(8–22)-GGG-NS5a(112–126)	$k' = 4.44$[a]		3371.71
cyc-HT	⌐TDWDVKGGGSPLYRHGD⌐	$k' = 3.36$[b]	1841.97	1842.9
cyc-CT	TDWDVKGGGSPLYRHGD⌐	$k' = 5.45$[a]		1841.7

[a] 10% B to 35% B in 30 min.
[b] 5% B to 60% B in 30 min.
[c] Kromasil C18 column (4.6 × 150 mm). Eluant A: 0.05% TFA in water. B: 0.05% TFA in acetonitrile. Flow rate at 1 ml min^{-1} and detection at 215 and 280 nm. Gradient elution.

Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Circular Dichroism

CD spectra were recorded at 5 °C in a Jasco 810 spectropolarimeter flushed with nitrogen (20 l min^{-1}). Measurements were carried out between 190 and 260 nm using a 1.0 mm path length cell. Data acquisition was performed at 10 nm min^{-1} using 1 nm as spectral band and 4 s as time constant. Data from three consecutive scans were averaged and processed to improve the signal-to-noise ratio. Peptide concentration (30–60 μM) was determined by quantitative amino acid analysis and spectrophotometrically at 280 nm. Peptide conformation experiments were carried out in aqueous buffer (5 mM Hepes, pH 7.4) and in the presence of structure-promoting solvents such as trifluoroethanol (TFE) or 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). CD spectra quantitative analysis were performed by Dichroweb server [25] and using the formalism of Chen *et al.* [26].

Fourier Transform Infrared Spectroscopy

A Bomem MB-120 Fourier transform infrared spectrometer equipped with DTGS detector was used for transmittance measurements on filters. FTIR spectra were measured with a resolution of 4 cm^{-1} and 50 scans were taken in order to obtain an appropriate signal-to-noise ratio without losing the signal. A CaF_2 flow cell and a window of 100 μm path length spacer were used. Peptide concentration was of 1.3 mM in D_2O, TFE and HFIP. Reference spectra of solvents were recorded in the same microcells and under identical instrument conditions as the sample spectra. Difference spectra were obtained by digitally subtracting the solvent spectrum from the peptide spectrum. Spectra deconvolution was carried out using Peakfit v 4.0 software.

RESULTS

Peptide Synthesis

The selection of the putative new epitopes from the GBV-C/HGV polyproteins was performed by the alignment of 34 published sequences of GBV-C/HGV isolates. The consensus sequences were obtained by means of a comparative study of GBV-C/HGV isolates using the Clustal W program (Figure 1), which is based on the prediction of antigenicity by analyzing the hydrophilicity and accessibility profiles of the proteins using the methods of Hopp and Woods [27] and Welling *et al.* [28]. Figure 2 shows the hydrophilicity and accessibility plots as well as the presence of β-turns that corresponds to NS4a, NS4b and NS5a GBV-C/HGV proteins.

The monomeric and chimeric peptides, whose primary sequences are shown in Table 1, were successfully synthesized by the robust solid-phase peptide methodology described in the Materials and Methods section. Yields based on a peptidyl-resin weight increase were almost quantitative. The purity of the samples after HPLC purification was checked by analytical RP-HPLC, in all cases being higher than 95%. Mass spectra showed the only presence of the expected molecular peaks (Table 1).

Since the NS4a(27–43) region is located in a β-turn or loop region of the protein (Figure 2), conformational restricted cyclic peptides were designed to mimic the native secondary structure better than the linear form, thus becoming more appropriate candidates for the development of synthetic antigens. The syntheses of cyclic constructs were successfully accomplished in the solid phase using an orthogonal three-dimensional protection scheme (Figure 3).

As previously described [29], the O-Dmab group is prone to a comparatively slow 1,6-elimination of the 4-aminobenzyl group following removal of the N-ivDde entity by 3% hydrazine. The presence of the 4-aminobenzyl ester precludes activation of the aspartic side-chain functionality and, consequently, the cyclization is not possible. In our hands, according to unpublished results (Chan, W.C., personal communication), to facilitate an effective and complete deprotection, after the hydrazine treatment, the peptide resin was exhaustively washed with 20% water in DMF. Intramolecular macrocyclization was then successfully achieved upon activation of the free resin-bound carboxy group by PyBOP.

The crude products were analyzed by RP-HPLC, which showed several peaks of similar intensities, indicating low reaction efficiency. The yields of the cyc-HT and cyc-CT after RP-HPLC purification were 4.4 and 4.8%, respectively.

Enzyme Immunoassays

In the present work and as we previously reported [17,30], in order to overcome the low coating efficiency of highly hydrophilic peptides to microtiter plates in the ELISA and to avoid unpredictable orientation of the peptides on the surface, the peptides were coupled covalently to the plates. Covalent binding, unlike simple physical binding, may orientate the immobilized peptides in a defined way on the solid phase. The most frequent methods of covalent immobilization of biomolecules on the solid phase involve either the preactivation of the biomolecules or the preactivation of the solid phase. In this work, different chemistries to link the peptides on the solid surface were studied with the purpose of selecting the best approach. Firstly, the peptides were immobilized via their amine groups. Secondly, after the activation with EDC/sulfo-NHS, the peptides were coupled to the solid phase by means of carboxy groups. Another approach involves the peptides coupling to preactivated *N*-oxysuccinimidyl active ester plates (Costar™) where the covalent immobilization is performed via the peptide amino groups. No significant statistical differences ($p > 0.05$) in the measured optical density were observed after the three assay formats performed with

Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

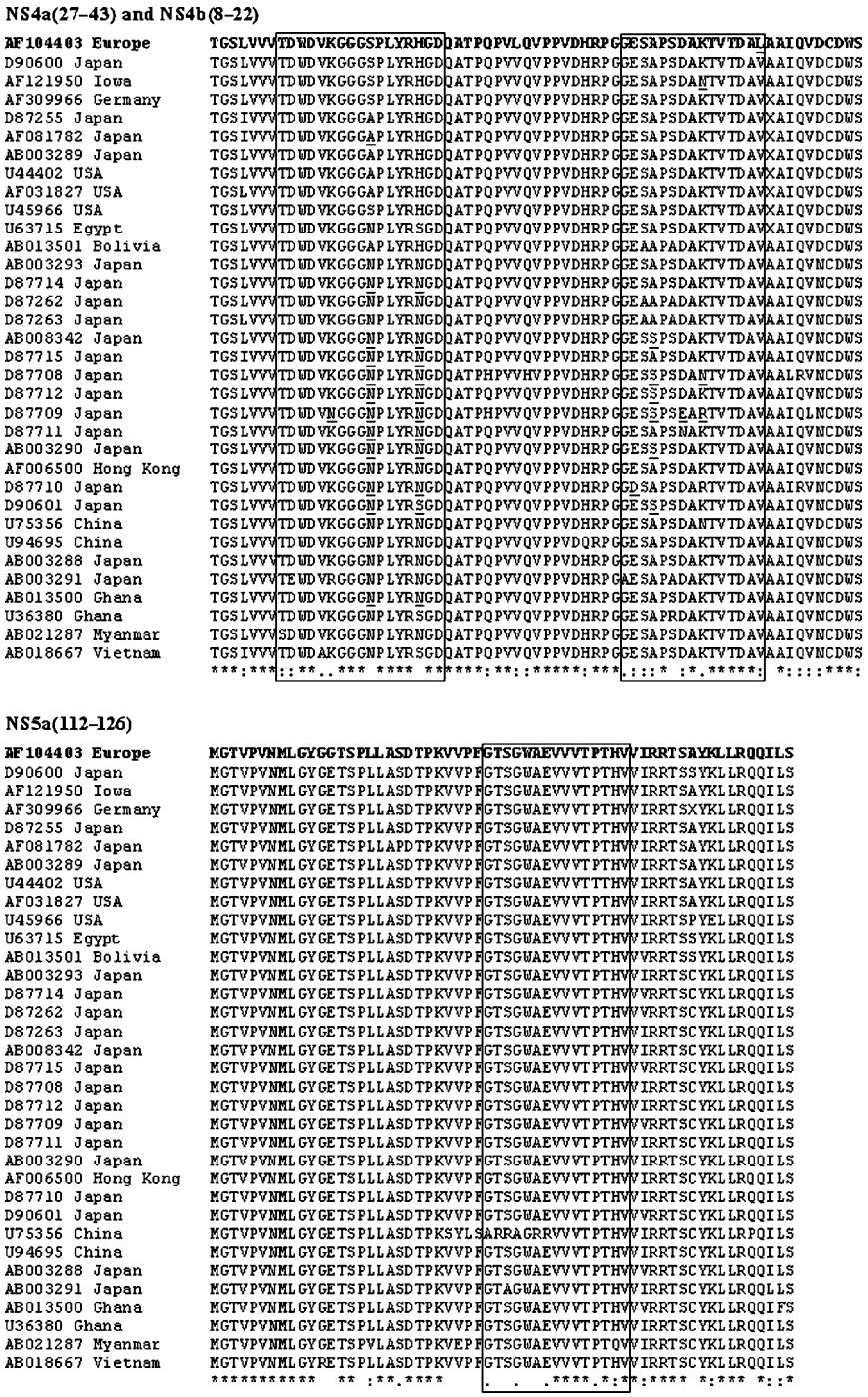

Figure 1 Alignment of the 34 sequences of GBV-C/HGV corresponding to NS4a(27–43), NS4b(8–22) and NS5a(112–126).

NS4b(8–22), NS5a(112–126) and chimeric peptides (data not shown); for this reason preactivated Costar ELISA plates were selected and extensively used to analyze the whole panels of sera.

ELISA results obtained while working with the 100 serum samples of panels of hemodialyzed and chronic hepatitis patients are shown in Figures 4 and 5. The samples were considered anti-GBV-C/HGV antibodies positive when (absorbance)Abs/CO ≥ 1.

In the case of the hemodialyzed panel (Figure 4), linear nonstructural sequences, i.e. NS4a(27–43), NS4b(8–22) and NS5a(112–126), and derived chimeric (Qm1 and Qm2) and macrocyclic (cyc-HT and cyc-CT) constructs exhibited a high number of serum samples scored as positive. Besides, our results indicate not only that the reactivities of the tests performed using chimeric and cyclic peptides were higher than those using the linear NS parent peptides but also that Abs/CO values tend to more clearly differentiate negative and positive sera, thus improving the detection of antibodies anti-GBV-C/HGV in serum. In this context, when Qm1 and Qm2 were used as coating antigens,

Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

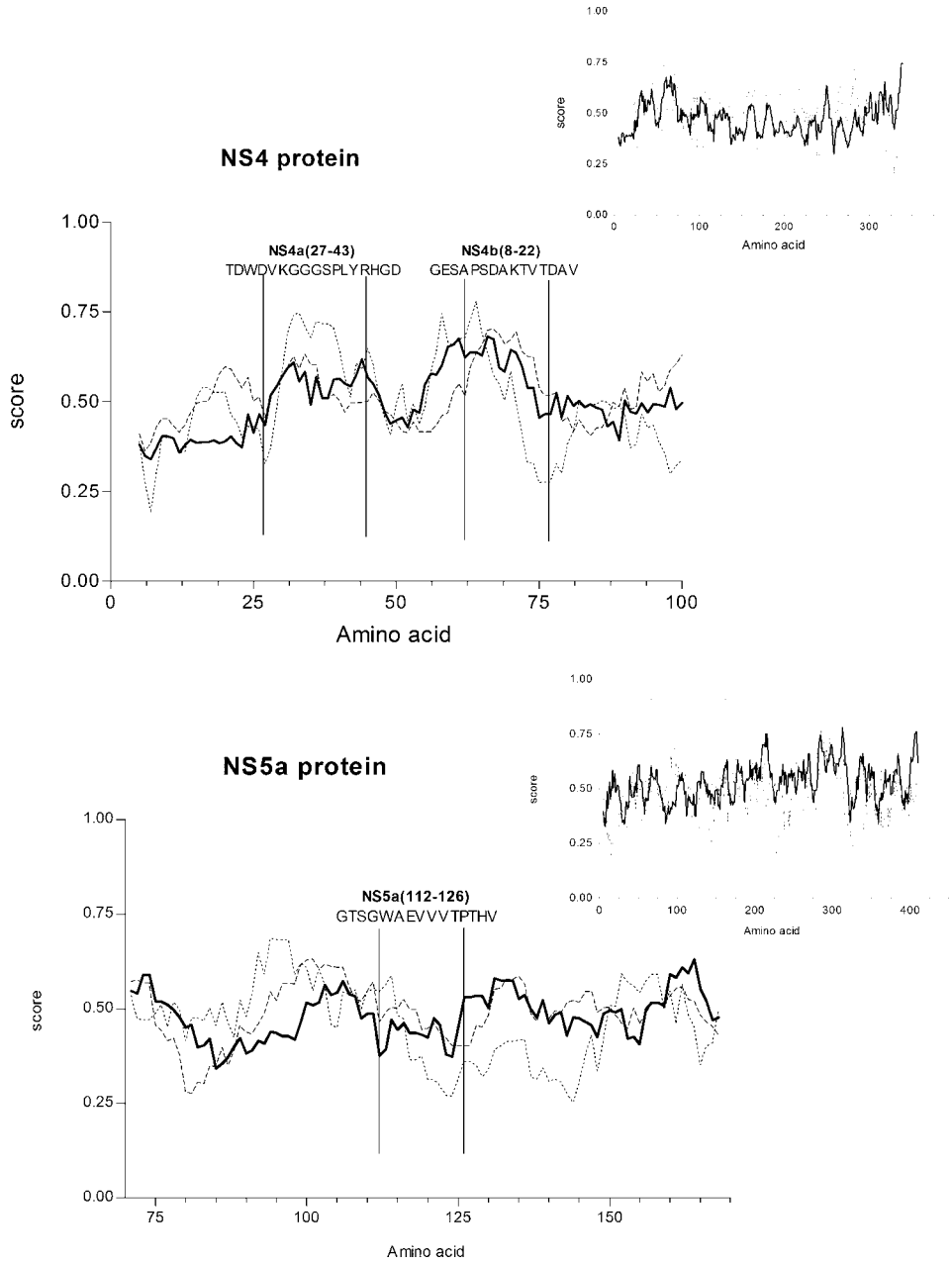

Figure 2 Hydrophilicity and accessibility plots corresponding to NS4 and NS5a proteins, using the Hopp & Woods (———), Welling (- - - -) and Chou & Fasman (·····) scales.

the number of serum samples giving Abs/CO > 1.2 was twice as those of the corresponding NS4b(8–22) peptide. For the NS5a(112–126) peptide, the serum reactivity was not so different. Similarly, when using macrocyclic constructs, 15 from the 23 (for cyc-HT) and 12 from 18 (for cyc-CT) scored positive sera rendered Abs/CO > 1.2, while for the parent peptide NS4a(27–43) only 7 serum samples were above this absorbance units. To sum up, chimeric and macrocyclic peptide constructions gave higher and clearer positive results.

In the case of the hepatitis chronic panel (Figure 5), the reactivity as well as the measured absorbance in positive samples were unexpectedly higher, when the linear NS5a(112–126) parent peptide was coupled to ELISA plates, than those obtained for NS4b(8–22), Qm1 and Qm2. In this case, more than 60% of the serum samples gave Abs/CO > 1.2. However, cyclic compounds again gave better reactivity and higher Abs/CO values than their parent peptide.

In Table 2, the ELISA results obtained with all the synthetic constructs and 35 sera are shown in comparison to the results obtained when using the commercial E2 recombinant protein-based test. As shown, for the linear sequences the percentage of peptide reactivity working with the positive anti-HGV recombinant E2 protein sera achieved values of about

Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

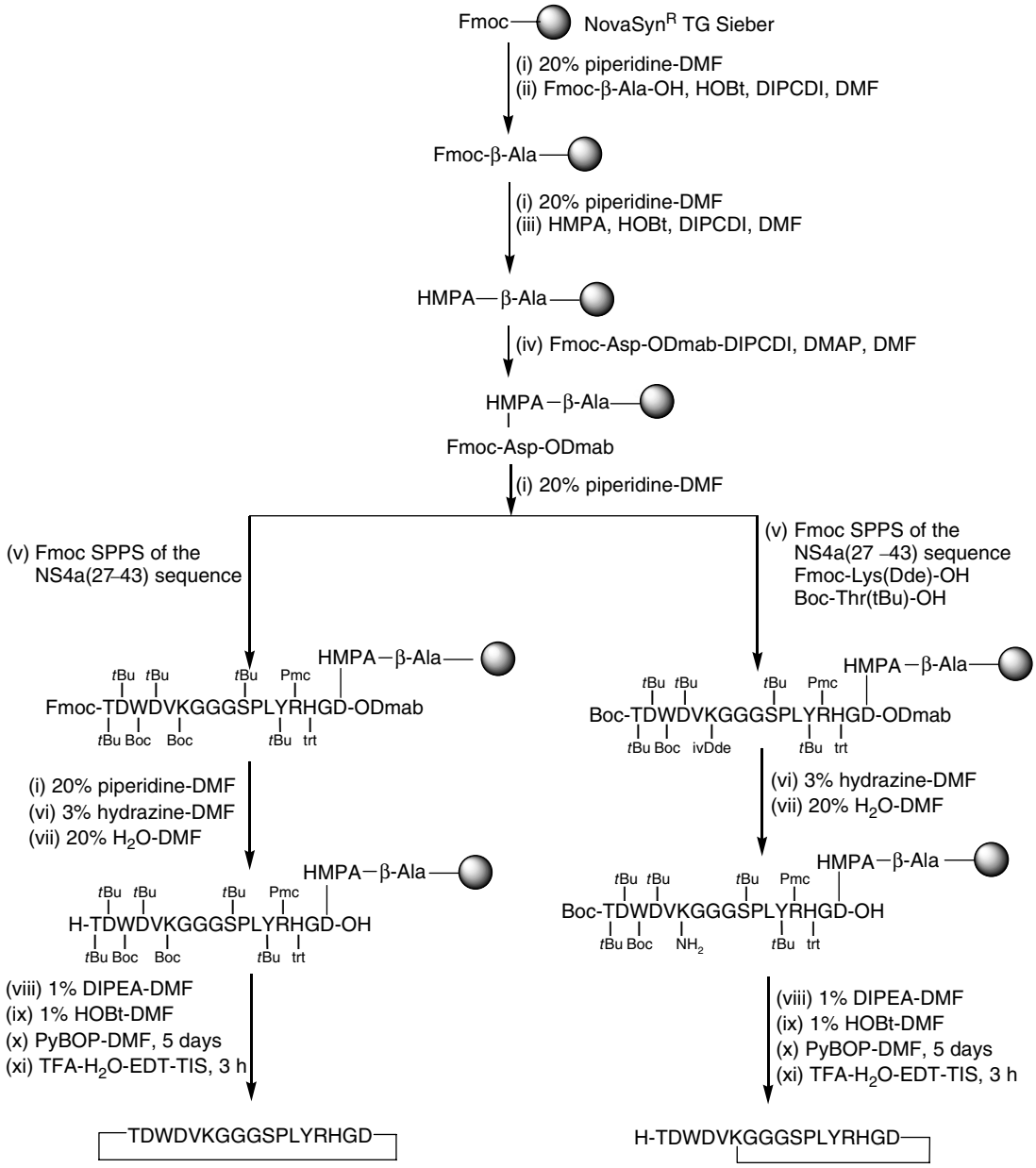

Figure 3 Strategy for solid-phase peptide synthesis of cyc-HT and cyc-CT cyclic peptides.

27% in the case of the hemodialyzed panel, whereas this value was only moderately higher (36%) for chimeric peptides. The reactivity obtained was similar when the negative anti-HGV recombinant E2 protein sera were studied for monomeric sequences; however, the number of positive samples detected by means of chimeric peptides was significantly higher, 10 of 24 for Qm1 (41%, $p = 0.009$) and 12 of 24 for Qm2 (50%, $p = 0.052$). Contrarily, in chronic hepatitis panel, reactivities till 8 and 15% for NS4b(8–22) and NS5a(112–126) monomeric sequences, respectively, were obtained while working with positive anti-HGV recombinant E2 protein-based tests, but no more than 15% of samples were positive for chimeric peptides. However, chimeric peptides were able to detect two times more negative anti-GBV-C/HGV E2 protein serum samples than monomeric peptides.

Regarding the behavior of cyclic peptides as antigenic substrates for detecting anti-GBV-C/HGV antibodies, as it is also shown in Table 2, the sensitivity of the two cyclic peptide variants was higher than those using the linear NS4a(27–43) peptide sequence. Specially, significant differences were observed in the reactivity of cyc-CT peptide compared to their parent sequence ($p < 0.05$).

Conformational Studies

Initial experiments carried out in Hepes buffer with all linear peptide constructs yielded spectra that exhibited a large negative band near 195 nm characteristic

Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

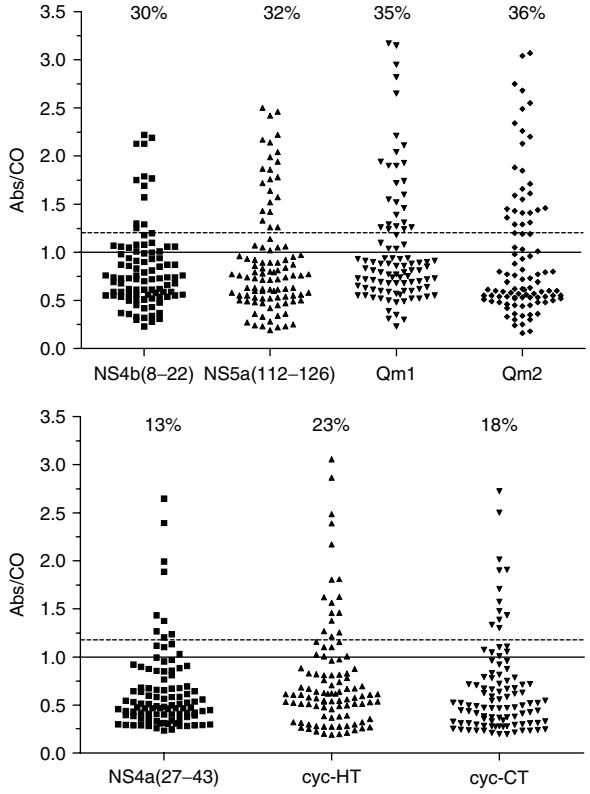

Figure 4 Reactivity in ELISA of the synthetic peptides with a hemodialyzed sera panel. The samples were considered anti-GBV-C/HGV antibodies positive when Abs/CO $\geq$ 1.

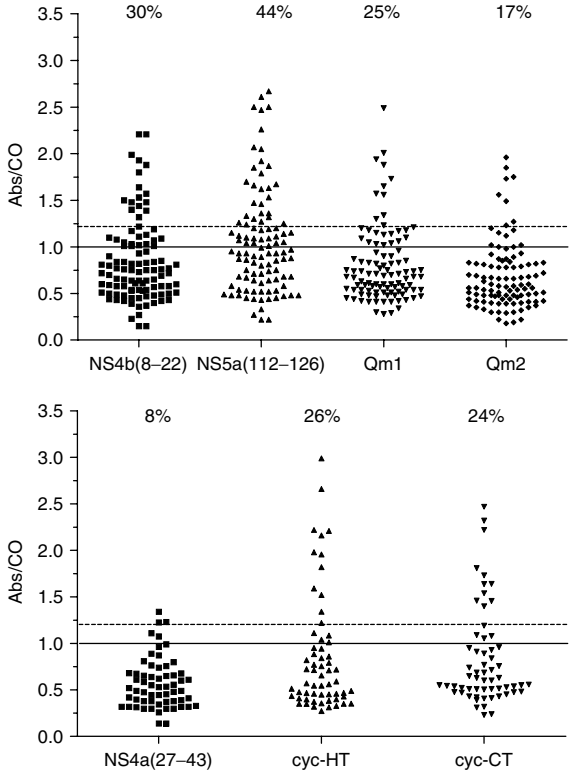

Figure 5 Reactivity in ELISA of the synthetic peptides with positive chronic hepatitis sera samples. The samples were considered anti-GBV-C/HGV antibodies positive when Abs/CO $\geq$ 1.

of unordered conformations. Next, the effect of the addition of halogenated alcohols (TFE and HFIP) was analyzed, the spectra obtained in the presence of both structure-promoting solvents being very similar (data not shown). As shown in Figure 6A, when the CD spectra of chimeric peptides in the presence of TFE was recorded, bands located at 201–206 and 222 nm tend to appear, indicating a mixture of conformers (α-helix and β-turn). As can be seen in the figure, for the two chimeric peptides, the n$\rightarrow \pi^*$ transition (222 nm) exhibits less intensity with respect to that of the $\pi \rightarrow \pi^*_{II}$ transition (208 nm) and tends to undergo a blue-shift. These small differences observed between the chimeric peptide spectra suggest that Qm1 tends to adopt a 3_{10} helix conformation, while the Qm2 peptide

Table 2 ELISA reactivity of E2 recombinant protein-based commercial test vs synthetic peptides NS4-NS5. Percentage of peptides reactivity in parenthesis

Hemodialyzed patients

	NS4b(8–22)	NS5a(112–126)	Qm1	Qm2	NS4a(27–43)	cyc-HT	cyc-CT
+anti-HG E2 protein ($n = 11$)	3/11 (27)	3/11 (27)	4/11 (36)	4/11 (36)	1/11 (9)	5/11 (45)	4/11 (36)
−anti-HG E2 protein ($n = 24$)	5/24 (21)	8/24 (33)	10/24 (41)	12/24 (50)	4/24 (17)	5/24 (21)	5/24 (21)

Chronic hepatitis patients

	NS4b(8–22)	NS5a(112–126)	Qm1	Qm2	NS4a(27–43)	cyc-HT	cyc-CT
+anti-HG E2 protein ($n = 13$)	1/13 (8)	2/13 (15)	1/13 (8)	2/13 (15)	1/13 (8)	1/13 (8)	2/13 (15)
−anti-HG E2 protein ($n = 23$)	2/23 (9)	1/23 (4)	4/23 (17)	2/23 (9)	1/23 (4)	2/23 (9)	2/23 (9)

Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

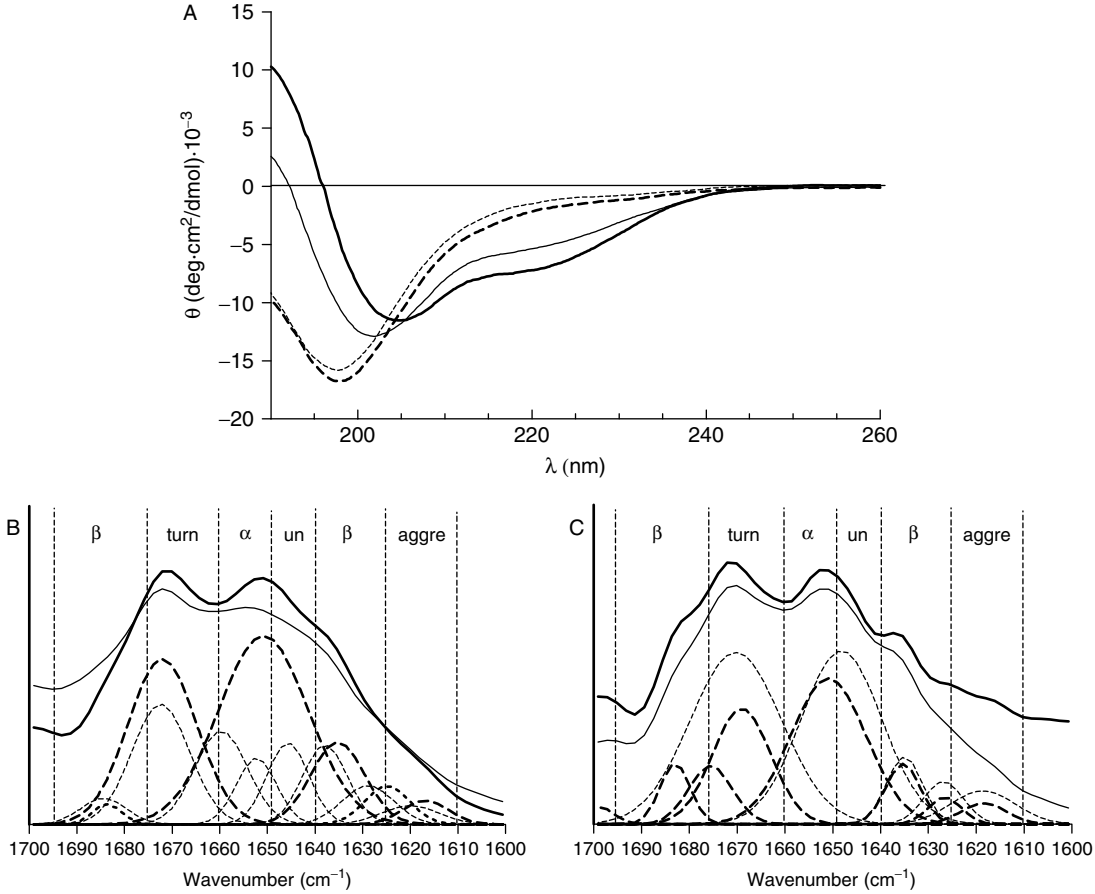

Figure 6 (A) CD spectra of chimeric peptides in aqueous buffer (Qm1: dash line; Qm2: bold dash line) and in presence of 75% of TFE (Qm1: solid line; Qm2: bold solid line). FTIR deconvolution of chimeric peptides in D_2O (B) and in 50% TFE (C). Qm1: solid line; Qm2: bold solid line. β: parallel and antiparallel sheets, α: α-helix, un: unordered, aggre: aggregated sheets/strands.

fits well with an α-helix conformation. The quantitative analysis of the experimental data performed by the measurement of θ at 222 nm and by the use of deconvolution computer programs showed that Qm2 adopted a larger proportion of α-helix structures than Qm1 (35 vs 23%). Furthermore, higher values for the $[\theta]_{222}/[\theta]_{\text{negmax}(\approx 205\text{ nm})}$ (R) ratio were obtained for Qm2 than for Qm1 peptide (0.60 vs 0.39). In this sense, values between 0.15 and 0.40 for R have been considered diagnostic for the 3_{10} helical conformation [31]. These results were supported by the FTIR analyses performed. As it is also shown in Figure 6, the characteristic bands assigned to α-helix and β-turn are obtained. It is noteworthy that while for Qm1 the amide I band located around 1650 cm^{-1}, which is assigned to an helical structure, only appeared in the presence of the α-helix promoting solvent (TFE) (Figure 6C), this component of the deconvoluted FTIR spectrum obtained for Qm2 was already registered in aqueous medium (47% content by the Peakfit software) (Figure 6B). Similar to the CD results obtained, in the presence of TFE, the two chimeric peptides tend to adopt a mixture of ordered conformations (43 and 39% for Qm1 and 46 and 28% for Qm2 contents of α-helix and β-turn, respectively).

In summary, these results suggest the predominance of a mixture of ordered structures for Qm1, while Qm2 has a higher tendency to clearly adopt an α-helix conformation. Moreover, the linear peptide conformation estimated by CD and FTIR were in agreement with each other.

Regarding the CD analysis of cyclic peptides (Figure 7) in an aqueous medium, cyc-HT showed a negative band below 200 nm, characteristic of unordered conformations or a mixture of multiple conformers, while the cyc-CT spectrum was more associated with folded conformations like turns, exhibiting the minimum below 200 nm, significantly less intensive than that observed for periodic-type secondary structures such as helices and sheets [32]. In the presence of 75% of TFE, the ellipticity of the band at 203 nm obtained for cyc-HT was higher than one of the bands at 222 nm, suggesting that this cyclic peptide tends to adopt preferably a 3_{10} helical structure, defined as an intermediate between helix and turn. However, in the same conditions, although there also appeared a blue-shifted

Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

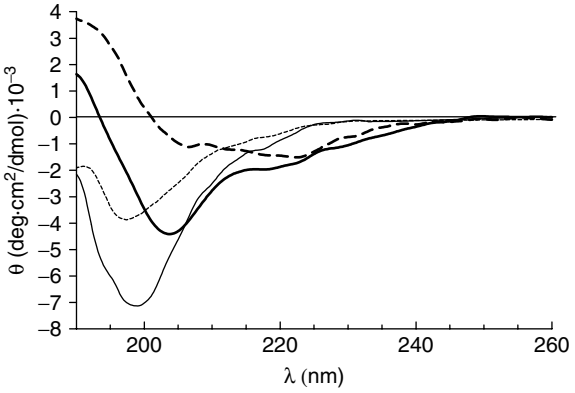

Figure 7 CD spectra of cyclic peptides in aqueous buffer (cyc-HT solid line; cyc-CT: dash line) and in presence of 75% of TFE (cyc-HT: bold solid line; cyc-CT: bold dash line).

band at 203 nm for cyc-CT of similar intensity than the band located at 222 nm, the lower ellipticity obtained compared to those in the CD-curve of cyc-HT suggest, preferably, the presence of folded structures in cyc-CT, like turns.

DISCUSSION

Several research groups have tried to identify, in recent years, well-defined immunodominant epitopes to diagnose infectious diseases using synthetic peptides. In our group, in order to establish the usefulness of synthetic peptides in the detection of antibodies anti-GBV-C/HGV, we initially determined the antigenicity of several peptide sequences belonging to structural and nonstructural proteins of this virus [17]. One of the main conclusions achieved was that the inclusion of nonstructural related peptides could increase the sensitivity of the currently available test for GBV-C/HGV detection that is based on the E2 structural protein.

Despite the fact that the role of the proteins encoded in nonstructural regions of the GBV-C/HGV viral genome is little-known, it is reported that the NS4a protein is a cofactor required for the NS3-mediated cleavage of some nonstructural proteins junction [33] and that the full-length NS4b is required for cleaving the NS4a/NS4b junction [34]. Related to the NS5 protein, it was found that the (2047–2376) region contains potential epitopes that elicit a detectable antibody response [35]. This property is rather similar to that in HCV infections, in which the antibodies to nonstructural proteins are easily detected [36,37].

Although synthetic peptides present high specificity in the recognition of antibodies, they have demonstrated limited sensitivity in immunoenzymatic assays when employed individually. In recent years, it has been reported that there was an improvement in sensitivity and specificity when sera were tested not only with monomeric but also with chimeric peptides which contain more than one putative epitope [13,38]. The use of mixtures of synthetic peptides could affect the sensibility and specificity of the test by the effect of competition for binding to solid surface and for changes in spatial distribution of antigenic determinants of bound peptides. In a previous study, we analyzed the ability of several synthetic peptides of the hepatitis A virus capsid proteins in comparison with the ones of a mixture of those peptides and with several chimeric constructs [13], the last approach rending the higher sensitivity. Similarly, in the present work, the results obtained using single, chimeric and a mixture of single peptides with several hemodialyzed sera revealed that the sensitivity of chimeric peptide-based methodology is again significantly higher (data not shown). As described in the Results section, chimeric peptides showed to be more sensitive for the hemodialyzed panel sera than the monomeric ones. However, the percentage of reactivity of chimeric peptides decreased working with the chronic hepatitis sera. These unexpected results may be explained either by cross-reactivity with antibodies directed against other viral proteins present in the patient sera like proteins of other hepatitis viruses of linear peptides or by an inadequate immobilization of chimeric peptides which could be responsible for the lower-than-expected antigen-antibody recognition. The first hypothesis, i.e. the elimination of the cross-reactivity by the inclusion of more than one single epitope in chimeric peptides, has already been described by other authors for the hepatitis C virus [39]. The validity of the second hypothesis was explored by performing inhibition ELISA tests – two negative sera for the chimeric peptides but positive for the linear NS4 and NS5 peptides were selected. Our results showed the ability of Qm1 and Qm2 to inhibit up to 25% of the NS4- and NS5-antibodies recognition, thus supporting the idea of the differential reactivity of chimeric constructs in solution, and hence a possible explanation for the low sensitivity obtained when performing in noncompetitive ELISA.

It is also noteworthy that the reactivity of cyclic peptides with the two panels of sera analyzed was significantly higher compared to the linear parent peptide. These results demonstrated that the constrained peptides could adopt conformations that favor antibody binding, i.e. the peptides may be presented in such a way that they easily adopt the conformational features of the original antigenic site of the native protein.

In addition, it is important to note that the reactivity of the peptides belonging to nonstructural proteins with the sera considered negative by the commercial test ranged between 17 and 21% for the hemodialyzed panel and, despite the chronic hepatitis panel these values were lower; the results obtained support the idea that the combination of antigens belonging to different viral proteins is necessary to ensure good sensitivity. In

Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

fact, and according to other studies [16], the sensitivity of currently available tests for GBV-C/HGV detection could be increased by the inclusion of nonstructural related peptides.

Finally, to analyze the conformational changes induced both in chimeric and cyclic peptides that could influence their different sensitivity in the GBV-C/HGV antibodies recognition, CD and FTIR studies were carried out and the corresponding quantitative analysis of the experimental data was done using deconvolution computer programs.

The experimental results obtained by CD and FTIR allowed us to disclose that, in general, a more ordered structure is related with a higher sensitivity in immunoassays. Although the ELISA assays performed with chimeric peptides did not show significant statistical differences, the significant variation in the antigenicity observed for linear and cyclic constructs suggest that the constrained peptides adopt a certain degree of structural organization that could be related with a better resemblance between the peptide epitope and the corresponding region within the whole viral protein.

The macrocyclization of the NS4a peptide to give the larger cyclic structure (cyc-HT) does not seem to provide sufficient conformational restriction since this peptide appeared to display a less ordered structure than cyc-CT. It is observed that the ring size reduction decreases the conformational repertoire; while cyc-HT fits well with a mixture of helix and turn (3_{10} helical conformation), cyc-CT preferentially adopts a more folded structure. However, although ELISA results clearly showed the usefulness of cyclization as an approach to enhance the antigenicity of linear peptides, nonsignificant differences between the two chemically engineered cyclic peptides were obtained, suggesting that the binding of peptides to the solid surface could probably cause changes in the epitope arrangement, making the access to the bioactive conformation difficult.

The results of the immunoassays reported highlight, on one hand, the combination of different antigens that seems to be necessary to ensure good sensitivity and more specificity and, on the other hand, that the cyclic compounds show higher ability to recognize anti-GBV-C/HGV antibodies than their parent peptide. Besides, they reflect the difficulty of achieving a high degree of conformational and chemical fit between the peptide antigen and the antibody surfaces.

We are currently investigating the synergistic effects of different presentation formats, and are therefore involved in the design of new chimeric and cyclic peptides bearing structural and nonstructural domains of GBV-C/HGV to better mimic the natural presentation of epitopes in the context of an *in vivo* antigenic situation.

Acknowledgements

This work was supported by the MCYT Project BQU2003-0507-CO2-02. Teresa Pérez acknowledges the CSIC I3P postgraduate scholarship program for financial support.

REFERENCES

1. Stapleton JT. GB virus type C/hepatitis G virus. *Semin. Liver Dis.* 2003; **23**: 137–148.
2. Halasz R, Weiland O, Sallberg M. GB virus C/hepatitis G virus. *Scand. J. Infect. Dis.* 2001; **33**: 572–580.
3. Siebert DJ, Bowden DS, Tracy SL, Hardikar W. Prevalence of hepatitis g/GBV-C in a healthy paediatric population. *J. Paediatr. Child Health* 2002; **38**: 423.
4. Chu C, Hwang S, Luo J, Wang Y, Lu R, Lai C, Tsay S, Li C, Wu J, Chang F, Lee S. Clinical, virological, immunological, and pathological significance of GB virus C/hepatitis G infection in patients with chronic hepatitis C. *Hepatol. Res.* 2001; **19**: 225–236.
5. Williams CF, Klinzman D, Yamashita TE, Xiang J, Polgreen PM, Rinaldo C, Liu C, Phair J, Margolick JB, Zdunek D, Hess G, Stapleton JT. Persistent GB virus C infection and survival in HIV-infected men. *N. Engl. J. Med.* 2004; **350**: 981–990.
6. Dille BJ, Surowy TK, Gutierrez RA, Coleman PF, Knigge MF, Carrick RJ, Aach RD, Hollinger FB, Stevens CE, Barbosa LH, Nemo GJ, Mosley JW, Dawson GJ, Mushahwar IK. An ELISA for detection of antibodies to the E2 protein of GB virus C. *J. Infect. Dis.* 1997; **175**: 458–461.
7. Tacke M, Schmolke S, Schlueter V, Sauleda S, Esteban JI, Tanaka E, Kiyosawa K, Alter HJ, Schmitt U, Hess G, Ofenloch-Haehnle B, Engel AM. Humoral immune response to the E2 protein of hepatitis G virus is associated with long-term recovery from infection and reveals a high frequency of hepatitis G virus exposure among healthy blood donors. *Hepatology* 1997; **26**: 1626–1633.
8. Thomas DL, Vlahov D, Alter HJ, Hunt JC, Marshall R, Astemborski J, Nelson KE. Association of antibody to GB virus C (hepatitis G virus) with viral clearance and protection from reinfection. *J. Infect. Dis.* 1998; **177**: 539–542.
9. Kunkel U, Hohne M, Berg T, Hopf U, Kekule AS, Frosner G, Pauli G, Schreier E. Quality control study on the performance of GB virus C/hepatitis G virus PCR. *J. Hepatol.* 1998; **28**: 978–984.
10. Tacke M, Kiyosawa K, Stark K, Schlueter V, Ofenloch-Haehnle B, Hess G, Engel AM. Detection of antibodies to a putative hepatitis G virus envelope protein. *Lancet* 1997; **349**: 318–320.
11. Dawson GJ, Chau KH, Cabal CM, Yarbough PO, Reyes GR, Mushahwar IK. Solid-phase enzyme-linked immunosorbent assay for hepatitis E virus IgG and IgM antibodies utilizing recombinant antigens and synthetic peptides. *J. Virol. Methods* 1992; **38**: 175–186.
12. Plagemann PG. Epitope specificity of monoclonal antibodies to the N-protein of porcine reproductive and respiratory syndrome virus determined by ELISA with synthetic peptides. *Vet. Immunol. Immunopathol.* 2005; **104**: 59–68.
13. Gomara MJ, Riedemann S, Vega I, Ibarra H, Ercilla G, Haro I. Use of linear and multiple antigenic peptides in the immunodiagnosis of acute hepatitis A virus infection. *J. Immunol. Methods* 2000; **234**: 23–34.
14. El Awady M, El Demellawy M, Khalil S, Galal D, Goueli S. Synthetic peptide-based immunoassay as a supplemental test for HCV infection. *Clin. Chim. Acta* 2002; **325**: 39–46.
15. Schellekens GA, de Jong BA, van den Hoogen FH, van de Putte LB, van Venrooij WJ. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. *J. Clin. Invest.* 1998; **101**: 273–281.
16. Toniutto P, Fabris C, Barbone F, Tisminetzky SG, Liani D, Galai T, Barillari G, Biffoni F, Gasparini V, Pirisi M. Immunoreactivity to putative B-cell epitopes of hepatitis G virus polyprotein in viremic and nonviremic subjects. *Clin. Diagn. Lab. Immunol.* 1999; **6**: 573–576.

Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

17. Rojo N, Ercilla G, Haro I. GB virus C (GBV-C)/hepatitis G virus (HGV): towards the design of synthetic peptides-based biosensors for immunodiagnosis of GBV-C/HGV infection. *Curr. Protein Pept. Sci.* 2003; **4**: 291–298.
18. Hernandez M, Selles ME, Pozo PL, Gomez I, Melchor A. Antigenicity of chimeric synthetic peptides based on HTLV-1 antigens and the impact of epitope orientation. *Biochem. Biophys. Res. Commun.* 2000; **276**: 1085–1088.
19. Drakopoulou E, Uray K, Mezo G, Price MR, Vita C, Hudecz F. Synthesis and antibody recognition of mucin 1 (MUC1)-alpha-conotoxin chimera. *J. Pept. Sci.* 2000; **6**: 175–185.
20. Dyson HJ, Wright PE. Antigenic peptides. *FASEB J.* 1995; **9**: 37–42.
21. Mezo G, Majer Z, Vass E, Jimenez MA, Andreu D, Hudecz F. Conformational study of linear and cyclic peptides corresponding to the 276–284 epitope region of HSV gD-1. *Biophys. Chem.* 2003; **103**: 51–65.
22. Schellekens GA, Visser H, de Jong BA, van den Hoogen FH, Hazes JM, Breedveld FC, van Venrooij WJ. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum.* 2000; **43**: 155–163.
23. Chan WC, Bycroft BW, Evans DJ, White PD. A novel 4-aminobenzyl ester-based carboxy-protecting group for synthesis of atypical peptides by Fmoc-but solid-phase chemistry. *J. Chem. Soc., Chem. Commun.* 1995; 2209–2210.
24. Cudic M, Wade JD, Otvos L. Convenient synthesis of a head-to-tail cyclic peptide containing an expanded ring. *Tetrahedron Lett.* 2000; **41**: 4527–4531.
25. Lobley A, Whitmore L, Wallace BA. Dichroweb: an interactive website for the analysis of protein secondary structure from circular dichroism data. *Bioinformatics* 2002; **18**: 211–212.
26. Chen YH, Yang JT, Chau KH. Determination of the helix and beta form of proteins in aqueous solution by circular dichroism. *Biochemistry* 1974; **13**: 3350–3359.
27. Hopp TP, Woods KR. Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. U. S. A.* 1981; **78**: 3824–3828.
28. Welling GW, Weijer WJ, van der ZR, Welling-Wester S. Prediction of sequential antigenic regions in proteins. *FEBS Lett.* 1985; **188**: 215–218.
29. Johnson T, Liley M, Cheeseright TJ, Begum F. Problems in the synthesis of cyclic peptides through use of the Dmab protecting group. *J. Chem. Soc. Perkin Trans. 1* 2000; 2811–2820.
30. Pérez T, Gómez A, Sanmarti R, Vi~nas O, Ercilla G, Haro I. Use of [Cit312,314] filaggrin (306–324) analogue for the diagnosis of rheumatoid arthritis. Conformational study by circular dichroism and Fourier transformed infrared spectroscopy. *Lett. Pept. Sci.* 2003; **9**: 291–300.
31. Formaggio F, Crisma M, Rossi P, Scrimin P, Kaptein B, Broxterman QB, Kamphuis J, Toniolo C. The first water-soluble 3(10)-helical peptides. *Chemistry* 2000; **6**: 4498–4504.
32. Perczel A, Hollosi M. Turns. In *Circular Dichroism and the Conformational Analysis of Biomolecules*, Fasman GD (ed.). Plenum Press: New York, 1996; 285–380.
33. Wright-Minogue J, Yao N, Zhang R, Butkiewicz NJ, Baroudy BM, Lau JY, Hong Z. Cross-genotypic interaction between hepatitis C virus NS3 protease domains and NS4A cofactors. *J. Hepatol.* 2000; **32**: 497–504.
34. Belyaev AS, Chong S, Novikov A, Kongpachith A, Masiarz FR, Lim M, Kim JP. Hepatitis G virus encodes protease activities which can effect processing of the virus putative nonstructural proteins. *J. Virol.* 1998; **72**: 868–872.
35. Pilot-Matias TJ, Muerhoff AS, Simons JN, Leary TP, Buijk SL, Chalmers ML, Erker JC, Dawson GJ, Desai SM, Mushahwar IK. Identification of antigenic regions in the GB hepatitis viruses GBV-A, GBV-B, and GBV-C. *J. Med. Virol.* 1996; **48**: 329–338.
36. Soffredini R, Rumi M, Lampertico P, Aroldi A, Tarantino A, Ponticelli C, Colombo M. Increased detection of antibody to hepatitis C virus in renal transplant patients by third-generation assays. *Am. J. Kidney Dis.* 1996; **28**: 437–440.
37. Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, Miyamura T, Dienstag JL, Alter MJ, Stevens CE. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science* 1989; **244**: 362–364.
38. Marin MH, Pena LP, Tanty CR, Higginson CD, Arenas MA, Noguerol KR, Leon CS. Antigenic activity of three chimeric synthetic peptides of the transmembrane (gp41) and the envelope (gp120) glycoproteins of HIV-1 virus. *Prep. Biochem. Biotechnol.* 2004; **34**: 227–237.
39. Rosa C, Osborne S, Garetto F, Griva S, Rivella A, Calabresi G, Guaschino R, Bonelli F. Epitope mapping of the NS4 and NS5 gene products of hepatitis C virus and the use of a chimeric NS4-NS5 synthetic peptide for serodiagnosis. *J. Virol. Methods* 1995; **55**: 219–232.

Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.