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Assessment of synthetic chimeric multiple antigenic peptides for diagnosis of GB virus C infection

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ABSTRACT

The use of synthetic peptides of both structural and nonstructural proteins of GB virus C (GBV-C) has been studied for the development of new systems to diagnose infection caused by this virus. In an attempt to increase the antigenicity of linear peptide sequences, chimeric multiple antigenic peptides (MAPs) containing epitopes from E2, NS4, and NS5 GBV-C proteins have been synthesized. The synthetic constructs were evaluated by ELISA to establish whether the epitopes in chimeric branched peptides are more efficiently recognized by the specific antibodies compared to the monomeric linear sequences. Moreover, we have investigated the application of a commercial biosensor instrument for the detection of antibodies against the GBV-C in human serum samples. The results of the immunoassays reported in this work highlight the usefulness of synthetic tetrameric branched peptides containing sequences from envelope and nonstructural GBV-C proteins for the diagnosis of GBV-C infection. The potential clinical value of the MAP_4(E2-NS5a) for the serodiagnosis of GBV-C infection was demonstrated, thus providing the basis for performing prevalence studies of the infection among the hemodialyzed and hepatitis C virus (HCV)-infected population.

The GB virus C (GBV-C)[1] (also formerly known as hepatitis G virus) is a virus made of a single chain of RNA that belongs to the Flaviviridae family. It is related to the hepatitis C virus (HCV) because it has a genomic structure and homology in the sequence very close to this hepatotropic virus. However, unlike HCV, it does not appear to cause hepatitis nor any other type of pathology [1,2].

There is clear evidence that GBV-C is transmitted by sexual and percutaneous routes and is frequently found in populations at risk for blood-borne or sexually transmitted viruses. Thus, GBV-C is more frequently detected in groups at higher risk for infection with hepatitis viruses by similar routes of transmission as well as in patients treated with multiple hemodialysis procedures and higher units of transfused blood products [3].

Particularly, GBV-C prevalence among patients with HCV monoinfection varies from 11% to 24% depending on the population studied [4–7]. GBV-C has been reported to have no impact on the outcome of HCV infection, HCV-related chronic liver disease, hepatic fibrosis, or transaminase levels [8]. However, in HCV/HIV-coinfected patients, the course of HCV disease is accelerated, with a considerable increase in progression of hepatic fibrosis [9]. Recently, Berzsenyi et al. [10] provided evidence of a significant reduction in HCV-related liver morbidity associated with GBV-C viremia in HCV/HIV-coinfected patients. In this sense, they pointed out that GBV-C infection may contribute to the unpredictable clinical outcomes in HCV/HIV-coinfected patients and could have a role as a prognostic marker in this setting.

Otherwise, it has been proposed that GBV-C might be a good indicator for demonstrating nosocomial parenteral transmission of viral agents [11]. Thus, GBV-C could be introduced as a good predictor for hospital-acquired infection as this virus is common in North America and Western Europe.

Although, there are currently no commercial systems to detect specific markers of GBV-C infection, active GBV-C infection has been detected by RT-PCR [12] while past infection has been detected by the presence of anti-E2 antibodies [13] using an ELISA assay involving the E2 recombinant protein. The development of an anti-E2 detection method has promoted a complete definition of the prevalence of GBV-C since E2 antibodies are several times more frequently detectable than RNA in blood donors [14]. Although the appearance of anti-E2 antibodies is considered to be an indication

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[1] *Abbreviations used*: AUC, area under the curve; BSA, bovine serum albumin; CM dextran, carboxymethyl dextran; DIPCDI, *N,N′*-diisopropylcarbodiimide; DMF, dimethylformamide; GBV-C, GB virus C; HCV, hepatitis C virus; HOBt, hydroxybenzotriazole; MAPs, multiple antigenic peptides; PBS, phosphate-buffered saline; ROC, receiver operating characteristic, TBTU, 2-(1*H*-benzotriazole-1-il)-1,3,3-tetramethyluronium-tetrafluoroborate; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; TIS, triisopropylsilane.

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of past infection, and detection of these antibodies and the active presence of GBV-C [15,16] are unlikely to be concomitant, recent literature provides descriptions of results that feature duality insofar as the positive presence of anti-E2 antibodies and the presence of viral RNA [17] are concerned.

On the other hand, it has been proposed that E2 antibodies are not a reliable marker of past GBV-C infection in populations with impaired immune function [18]. Particularly, HCV-coinfected patients receiving interferon therapy can also eliminate the virus without the development of anti-E2 antibodies. Thus, the elimination of the infection without antibodies against E2 protein raised questions about the usefulness of E2 antibodies as a marker of past GBV-C infection among HIV or HIV–HCV-coinfected patients.

The above noted results point to the need for further studies on the development of the antibodies to provide an understanding of the effect of exposure to GBV-C on human health in the context of other viral infections.

In our group, the use of synthetic peptides of both structural and nonstructural proteins of GBV-C has been studied for the development of new systems to diagnose infection caused by this virus. Our work has mainly involved the use of chemically well-defined antigens that guarantee less inter- and intra-assay variability. Hence, epitopes have been localized in E1 and E2 structural and NS3, NS4, and NS5 nonstructural proteins of GBV-C and new linear, chimeric, and cyclic antigens have been designed [19–21]. Our results have shown that the combination of different antigens is clearly necessary to achieve both a good degree of sensitivity in the immunoenzymatic assay and a greater specificity in results.

In an attempt to increase the antigenicity of previously reported linear peptide sequences, chimeric multiple antigenic peptides (MAPs) containing epitopes from E2, NS4, and NS5 GBV-C proteins have been synthesized. The synthetic constructs were evaluated by ELISA to establish whether the epitopes in chimeric branched peptides are more efficiently recognized by the specific antibodies compared to the monomeric linear sequences. Moreover, we have investigated the application of a commercial surface plasmon resonance-based biosensor instrument for the detection of antibodies against the GBV-C in human serum samples.

Experimental

Peptide synthesis

Multiple synthesis of 24 linear peptides related to the domain (89–149) of the E2 envelope protein of GBV-C was carried out in parallel in the solid phase using a semiautomatic synthesizer (Multisyntech GmbH). The 15-mer peptides are overlapped sequences by 13 residues. A Rink amide resin (TentaGel R RAM, RAPP Polymere GmbH) of functionalization 0.2 mmol/g, which allows carboxamide peptides to be obtained at the C-terminal end, was used. The Fmoc/*t*Bu strategy based on the use of the 9-fluorenylmethoxycarbonyl (Fmoc) group, labile to piperidine/DMF, was used as temporary protection for the α-amino functions and *tert*-butyl (*t*Bu) groups, labile to 95% trifluoroacetic acid (TFA), as semipermanent protection for the different functions of the side chains of the amino acids (*t*Bu esters or ethers). The reagent groups were activated essentially by means of treatment with *N*,*N*′-diisopropylcarbodiimide (DIPCDI) and hydroxybenzotriazole (HOBt). The coupling reaction was carried out in duplicate using threefold excess of reagents. For the second coupling, the carboxyl group was activated by means of addition of a phosphonium salt, benzotriazole-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBop), in the presence of HOBt and a base such as diisopropylethylamine (DIEA).

Once the synthesis was complete, the cleavage and deprotection process of the peptidyl resins was carried out in a semiautomatic synthesizer using the Multisyntech accessories available for this purpose. This reaction was taken place by means of treatment with 95% TFA in the presence of scavengers, basically H_2O and triisopropylsilane (TIS).

The peptides were characterized by analytical high performance liquid chromatography (HPLC) and electrospray mass spectrometry.

The syntheses of MAP_4(E2-NS5a) and MAP_4(E2-NS4a) were carried out manually in a Rink amide resin (NovaSyn TGR, NovaBiochem) of functionalization 0.29 mmol/g. Amino acid side chain protection was effected by the following: trityl (triphenylmethyl) for glutamine, asparagine, and histidine; *tert*-butyl for aspartic acid, glutamic acid, serine, and threonine; 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for arginine; Fmoc or 1-(4,4-dimethyl-2,6-dioxocyclohexylidene) 3-methyl-butyl (Dde) and *tert*-butoxycarbonyl (Boc) for tryptophan. The Cys residues present in the E2(99–118) at positions 112 and 114 were replaced by 2-aminobutyric acid (Abu) residues. This amino acid has the same hydrophobic and steric properties as cysteine, but avoids the reactivity of the Cys thiol group without modifying the immunochemical properties of the parent peptide [22].

The first amino acid coupled to the resin was β-Ala. The tetravalent lysine core was obtained by sequential coupling of 0.4 mmol of Fmoc-Lys(Fmoc)-OH and 0.8 mmol of Fmoc-Lys(Dde)-OH, which were incorporated through 2-(1*H*-benzotriazole-1-il)-1,3,3-tetramethyluronium-tetrafluoroborate (TBTU):HOBt:DIEA (1:1:2)-mediated carboxyl activation. The assembly of the peptide sequences E2(99–118) or E2(7–26) was then accomplished at both Nα-lysine positions. An eightfold molar excess of Fmoc-amino acids, DIPCDI/HOBt was used throughout the synthesis. The N-terminal amino acid was introduced as a *tert*-butoxycarbonyl (Boc) Nα-protected residue.

The *N*ε-Dde-protecting group was removed by treatment with 2% hydrazine in DMF [23]. The assembly of the NS5a(112–126) or NS4a(27–43) sequences was then performed at both exposed lysine Nε-amino groups of the monoepitopic MAP resin intermediates. For difficult couplings TBTU and DIEA agents were used. The efficiency of these reactions was evaluated by the kaiser (ninhydrin) test and repeated couplings were carried out when a positive ninhydrin test was observed.

The diepitopic MAPs were concomitantly side chain deprotected and cleaved from the resins by treatment with a mixture of TFA in the presence of TIS and water as scavengers (TFA:TIS:H_2O, 9.5:0.25:0.25) for 6 h with occasional agitation at room temperature. Then, the resin was filtered and washed with TFA. The filtrates were evaporated in vacuum to dryness and the crude peptides were precipitated with diethyl ether to afford white solids, which were collected, dissolved in 30% acetic acid in water, and lyophilized.

MALDI-TOF mass spectrometry spectra confirmed the expected molecular mass of the diepitopic MAPs: [MH+] = 7899.9 and [MH+] = 8356.8 for MAP_4(E2-NS5a) and MAP_4(E2-NS4a), respectively. Purification of the crude molecules was achieved by semipreparative HPLC on a Kromasil C18 column (250 × 4.6 mm).

The synthesis of the linear peptides E2(7–26), GSRPFEPGLTWQ SCSCRANG; NS4a(27–43), TDWDVKGGGSPLYRHGD; NS4b(8–22), GESAPSDAKTVTDAVD; NS5a(112–126), GTSGWAEVVVTPTHVD; Qm1 [NS5a(112–126)-GGG-NS4b(8–22)], GTSGWAEVVVTPTHVDG GGGESAPSDAKTVTDAVD; and Qm2 (NS4b(8–22)-GGG-NS5a(112–126)), GESAPSDAKTVTDAVDGGGGTSGWAEVVVTPTHVD has been described previously [20].

Serum specimens

Three panels of serum samples from the Hospital Clinic of Barcelona were studied. The first and second groups consisted of 95

sera from hemodialyzed patients and 65 sera from chronic hepatitis patients, respectively. The third group consisted of 50 control sera from volunteer blood donors. Thirty-five samples of the hemodialyzed and 36 samples of the chronic hepatitis panels were tested for the presence of anti-HGV E2 protein (Roche Diagnostics + GmbH).

Enzyme linked immunosorbent assay (ELISA)

Peptides were coupled to assay plates (Costar Corp. DNABind *N*-oxysuccinimide surface) at 1 μg per well. Coupling was performed overnight at room temperature at pH 9.6 in carbonate/bicarbonate buffer (0.05 M).

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 evaluate nonspecific reactions of sera. For blank controls, wells were coupled with BSA (2 μg/well). After incubation, the plates were blocked with 2% BSA in carbonate/bicarbonate (0.05 M, pH 9.6) buffer for 1 h at room temperature. Sera were diluted 50-fold in RIA buffer (1% BSA, 350 mM NaCl, 10 mM Tris–HCl, pH 7.6, 1% vol/vol Triton X-100, 0.5 wt%/vol sodium deoxycholate, 0.1% SDS) supplemented with 10% fetal bovine serum. An amount of 100 μl/well was added, and the mixture was incubated for 1.5 h at room temperature. After the mixture was washed six times with PBS/0.05% Tween 20, 100 μl/well of antihuman IgG conjugated to peroxidase diluted in RIA buffer (1:1000) was added. After incubation for 1 h at room temperature, the plates were washed six times with PBS/0.05% Tween 20 and bound antibodies were detected with *o*-phenylenediamine dihydrochloride (OPD, Sigma Chemical Company) and 30% hydrogen peroxide (0.8 μl/10 ml). The plates were incubated at room temperature for 30 min. The reaction was stopped with H_2SO_4 (2 N, 50 μl) per well, and absorbance values were measured at a wavelength of 492 nm. All sera were tested in duplicate. Control sera were also included to monitor inter- and intra-assay variations.

Biospecific interaction analysis (BIA)

The Biacore T100 analytical instrument, CM5 sensor chip, and the running buffer (HSB-EP) containing 10 mM HEPES, pH 7.4, NaCl 150 mM, EDTA 3.4 mM, and 0.05% P20 surfactant were provided by Biacore (GE Healthcare).

Prior to peptide immobilization on the CM5 chip, optimal conditions for diffusion into the hydrophilic matrix were obtained. Different pH values (ranging from 3.1 to 5, depending on the p*I* of the synthetic construct) and different peptide concentrations (ranging from 12.6 nM to 1.26 μM) were assayed in order to optimize the signal for capture of anti-GBV-C/HGV antibodies (data not shown). Immobilization was performed at 25 °C and at a flow rate of 10 μl/min in HBS-EP as a running buffer. The sensor chip CM5 was activated by injecting *N*-hydroxysuccinimide (NHS) (0.05 M) and *N*-ethyl-*N*′-(3-diethylaminopropyl)-carbodiimide (EDC) (0.2 M) (1:2, v:v). Peptides were then covalently coupled to the sensor chip through reaction with primary amines. Excess reactive groups were deactivated by injecting 50 μl of 1 M ethanolamine adjusted to pH 8.5. Thus, 0.17, 0.19, 0.14, and 0.11 pmol/mm^2 of NS4b(8–22), NS5a(112–126), Qm1 (NS5a(112–126)-GGG-NS4b(8-22)), and Qm2 (NS4b(8–22)-GGG-NS5a(112–126)), respectively, were immobilized in different flow cells. Moreover, 0.034, 0.091, and 0.24 pmol/mm^2 of diepitopic MAP were immobilized in three different flow cells of one CM5 chip. Control experiments were performed using sensor chips activated according to the protocol described but without any peptide coupled.

Sera were diluted 1/50, 1/100, and 1/200 in running buffer containing 0.1% CM dextran (CM dextran). Samples were injected at 25 °C into the flow cells at a flow rate of 10 μl/min using HBS-EP as running buffer. The contact time between a sample and the specific surface was 10 min. The same peptide surface was used repeatedly to study several samples. This was accomplished by washing away the associated antibodies by an injection of 1 min pulse of NaOH (100 mM) between tests. To determine whether peptide–antibody binding had occurred, the cutoff of the analysis was calculated for each peptide relative to negative control sera (mean + 2 standard deviations).

Statistical analysis

Receiver operating characteristic (ROC) curve analysis, Mann–Whitney *U* test was conducted using MedCalc version 7.6 (MedCalc Software, Mariakerke, Belgium). Samples were considered positive when the absorbance was equal to or higher than the CO. The CO value was calculated from the ROC analysis.

The reactivities of each peptide construct with the panel of sera were compared using the *t* test analysis. Probability values less than 0.05 were considered statistically significant.

Results and discussion

Selection of antigenic peptide sequences

There is currently great interest in work on the design of peptidic sequences that are three dimensionally similar to the most significant protein epitopes and meanwhile ensure total homogeneity of the antigen used in immunoassays. A number of approaches, including experimental and computational, have been described to select peptides that functionally mimic B-cell epitopes at the level of single amino acids. In an attempt to design a chimeric multiple antigen peptide as a better antigenic peptide that could improve the sensitivity and specificity of immunoassays based on linear monomeric peptides, we have selected the epitopes taking into account both previous studies carried out in our group and epitope mapping of a selected E2 region.

We have previously reported the usefulness of E2(99–118) and E2(125–139) peptides for the diagnosis of GBV-C infection [21]. In the present work, in order to better comprehend the GBV-C sera reactivities with E2 peptides, we proceeded to analyze the amino acid requirements by synthesizing 15-mer overlapped peptides in 12 residues that scan the (89–149) region of GBV-C E2 protein. The peptides that are shown in Table 1 were successfully synthesized by multiple synthesis and characterized by analytical HPLC and electrospray mass spectrometry (ES-MS). Considering that viral hepatitis is highly prevalent in the dialysis population and patients in maintenance hemodialysis have high risk of GBV-C infection as well as patients coinfected with HCV, antibody reactivity to these synthetic peptides was assessed in a cohort of hemodialyzed and chronic C hepatitis patients. These peptides were analyzed in a direct binding assay (ELISA test) to evaluate their recognition by the antibodies present in both panels of sera. To identify the best recognized epitope, the 24 peptides were first assayed with 8 hemodialyzed and 8 chronic C hepatitis patient sera and 4 samples of serum from healthy blood donors. As shown in Fig. 1, an increase in sensitivity was seen predominantly for P6, P7, P8, and P9 peptides. Moreover, the region corresponding to P18 and P19 peptides also rendered a good performance in the ELISA assay. Nevertheless, a lower specificity of this last region compared to the P6–P9 sequences was obtained, the reactivity measured against blood donor sera being clearly higher for P18–P19 peptides (Table 1, supplementary material). Taking into account these results, the (99–118) sequence of E2 protein corresponding to P6–P9 peptides was selected as an antigenic peptide domain to be included in the

Table 1
Primary structure and ES-MS data of synthetic peptides that scan the (89–149) region of GBV-C E2 protein.

	E2 (99-118) / E2 (125-139)	ES-MS	
	SVSVTCVWGS**VSWFASTGGRDSKIDVWSLV**PVGSAS**CTIAALGSSDRDTVV**ELSEWGVPCA	Observed masses	Expected masses
P1	SVSVTCVWGS**VSWFA**	1613.75	1613.84
P2	SVTCVWGS**VSWFAST**	1615.73	1615.81
P3	TCVWGS**VSWFASTGG**	1543.68	1543.70
P4	VWGS**VSWFASTGGRD**	1610.75	1610.73
P5	GS**VSWFASTGGRDSK**	1540.73	1540.64
P6	**VSWFASTGGRDSKID**	1624.78	1624.76
P7	**WFASTGGRDSKIDVW**	1723.83	1723.89
P8	**ASTGGRDSKIDVWSL**	1590.80	1590.74
P9	**TGGRDSKIDVWSLV**P	1628.85	1628.83
P10	**GRDSKIDVWSLV**PVG	1626.87	1626.86
P11	**DSKIDVWSLV**PVGSA	1571.82	1571.78
P12	**KIDVWSLV**PVGSAS**C**	1559.80	1559.83
P13	**DVWSLV**PVGSAS**CTI**	1532.75	1532.76
P14	**WSLV**PVGSAS**CTIAA**	1460.73	1460.70
P15	**LV**PVGSAS**CTIAALG**	1357.73	1357.62
P16	PVGSAS**CTIAALGSS**	1319.64	1319.48
P17	GSAS**CTIAALGSSDR**	1394.65	1394.51
P18	AS**CTIAALGSSDRDT**	1466.67	1466.57
P19	**CTIAALGSSDRDTVV**	1506.73	1506.68
P20	**IAALGSSDRDTVV**EL	1544.80	1544.71
P21	**ALGSSDRDTVV**ELSE	1576.76	1576.67
P22	**GSSDRDTVV**ELSEWG	1635.74	1635.69
P23	**SDRDTVV**ELSEWGVP	1687.81	1687.81
P24	**RDTVV**ELSEWGVPCA	1659.79	1659.86

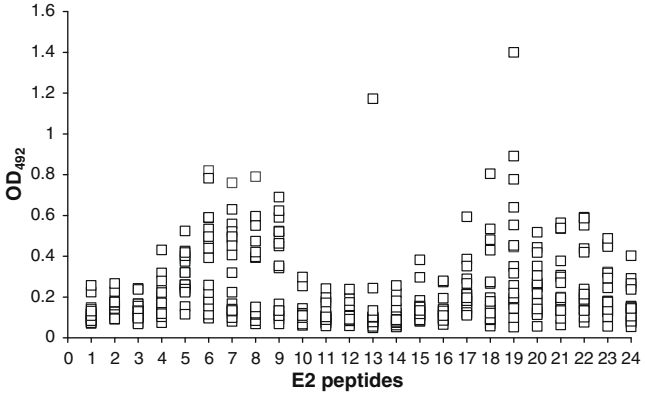

Fig. 1. Reactivity of hemodialyzed and chronic hepatitis patient sera with 24 overlapped peptides that scan the (89–149) region of GBV-C E2 protein.

tetrameric structure of one chimeric MAP. Besides, the NS5a(112–126) peptide was also selected according to the results previously obtained [20]. As described, the reactivity of the NS5a(112–126) peptide with the sera considered negative by the commercial E2 recombinant-protein test was quite significant, supporting the idea that the combination of antigens belonging to different viral proteins is necessary to ensure good sensitivity. Thus, MAP_4[E2(99–118) and NS5a(112–126)] was decided to be tested for reactivity in the above described panels of sera.

On the other hand, to further analyze the usefulness of MAP constructs and to investigate the antigenic domain dependence of the antibody response, MAP_4[(E2(7–26), NS4a(27–43)] was also selected for synthesis. This chemical structure combines a linear peptide corresponding to the N-terminus of the E2 protein which was demonstrated to be highly reactive against sera containing anti-GBV-C antibodies (results to be published), and a previously defined antigenic region of the NS4a protein [20] .

Reactivity of chimeric multiple antigenic peptides by ELISA

As previously described, a useful attempt to increase the antigenicity of peptide sequences is the covalent attachment of epitopes to synthetic branched polymeric scaffolds of controlled chemical composition [24]. In this sense, the MAP system based on a small immunogenically inert core matrix of lysine residues bearing radially branching synthetic peptides has been described as a multimeric form that improves the antigenic capacity of monomeric linear peptides. The main advantages of MAP constructs are that they consist of pure antigens and provide scaffolding for close packing of peptide antigens which permit stabilization of the secondary structure and reverse turns of peptide antigens. MAPs of different complexity have been used for detection of specific antibodies of human diseases such as HIV-I infection [25,26], hepatitis A [27], allergy [28], herpes simplex virus (HSV) infection [29], human herpes virus (HHV) infection [30], and systemic lupus erythematosus [31].

In this work, the synthesis of two heterogeneous and tetrameric macromolecular constructs was successfully accomplished using a combination of orthogonal Dde amino protection and Fmoc-based solid-phase chemistry [32]. The structure of the diepitopic MAPs that are based on a tetrameric lysine core is represented in Fig. 2. The crude peptides were purified by semipreparative reversed-phase HPLC and the identity of the purified synthetic protein constructs was confirmed by MALDI-TOF and electrospray MS analysis (Fig. 1, supplementary material).

With the aim of investigating the synergistic effects of presenting two copies of epitopes from different GBV-C proteins in the same macromolecule, antibody reactivity to the synthetic MAPs were assessed in a cohort of 95 hemodialyzed and 65 chronic C hepatitis patient sera. Fifty sera from healthy blood donors were used as control.

Diepitopic MAP containing two copies of E2(99–118) and NS5a(112–126) epitopes was compared with the corresponding

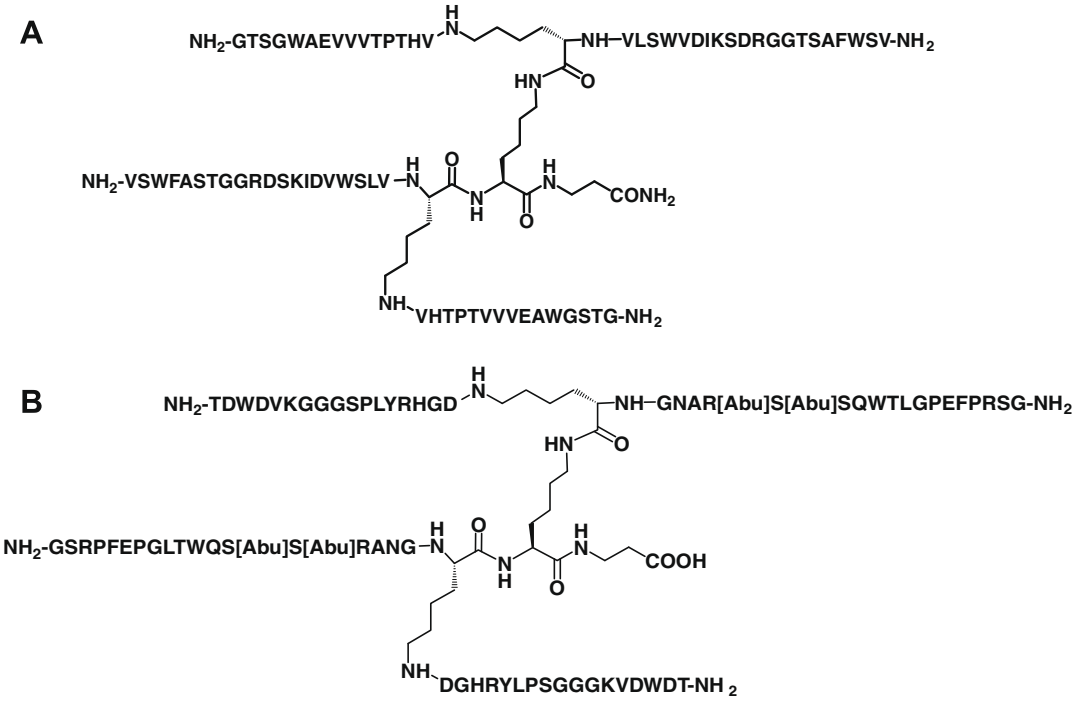

Fig. 2. Primary structure of: (A) MAP_4[E2(99–118)-NS5a(112–126)] and (B) MAP_4[E2(7–26)-NS4a(27–43)]. Abu: L-α-aminobutyric acid.

monomeric peptides as coating antigens in the ELISA. As shown in Fig. 3, the ROC curve analysis showed higher area under the curve (AUC) value for MAP_4(E2-NS5a) (0.874) compared to the linear monomeric peptides (0.719 and 0.751 for NS5a(112–126) and E2(99–118), respectively). The higher specificity obtained for the MAP compared with the monomeric linear peptides as well as the increase in the sensitivity indicates that this multimeric peptide bearing peptide sequences from envelope and nonstructural GBV-C proteins within the same molecule is more antigenic than the corresponding parent peptides and can be used to detect specific anti-GBV-C antibodies to more than one epitope simultaneously.

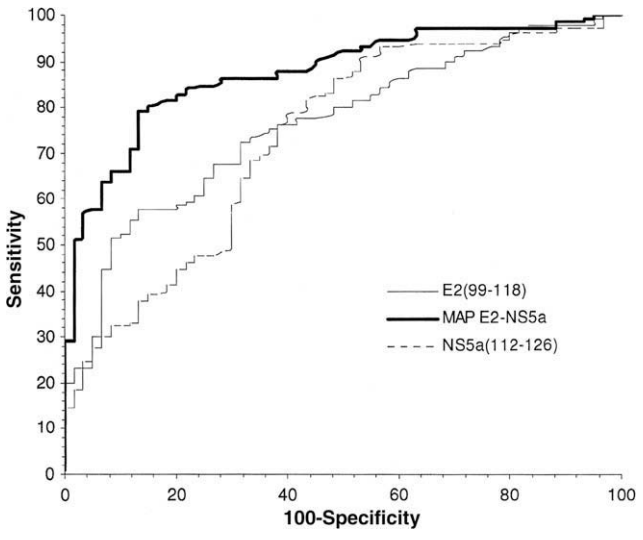

Fig. 3. ROC curve analysis for E2(99–118), NS5a(112–126), and MAP_4(E2-NS5a) antibody reactivity in the cohort of both hemodialyzed and chronic hepatitis patient sera. Sensitivity and specificity were calculated for all potential cutoff values and plotted ROC curves ($n = 95$ hemodialyzed and 65 chronic hepatitis patients, $n = 50$ blood donors). The area under the ROC curves was 0.751, 0.719, and 0.874 for E2, NS5a, and MAP E2-NS5a, respectively.

Considering the success of the results obtained with the above described MAP construct, the antigenic capacity of the MAP_4(E2-NS4a) was also analyzed with the same panels of sera. In Fig. 4 the relationship between the sensitivity and the specificity of the assays performed with the two diepitopic MAP constructs for different cutoff values is presented graphically by means of the ROC curve analysis. We could observe a significantly higher area under the curve value for the synthetic MAP_4(E2-NS5a) (AUC = 0.806 and 0.947) compared to the MAP_4(E2-NS4a) (AUC = 0.626 and 0.764) for both panels of sera, hemodialyzed and chronic hepatitis patients, respectively. Fig. 4b shows the reactivity of both MAP_4(E2-NS5a) and MAP_4(E2-NS4) with three panels of sera, hemodialyzed, chronic C hepatitis, and blood donors, respectively. The cutoff selected from the ROC curve results corresponded to 98% specificity. The reactivities of the MAP_4(E2-NS5a) for hemodialyzed and chronic C hepatitis patient sera were significantly higher than those of the MAP_4(E2-NS4).

These results clearly indicate that the presence of peptides belonging to the NS5a nonstructural protein is necessary to ensure a good balance sensitivity/specificity.

In the development of tests to identify antibodies to HCV the addition of antigens from the NS5 region in the third generation ELISA assays improved the sensitivity of the tests [33]. The reactivity to NS5 proteins can also be identified individually by supplementary assays, as recombinant immunoblot assay (RIBA), which detects antibodies to each of the HCV proteins in a support membrane. Furthermore, antibodies to HCV-NS5 in HCV infection may be a surrogate marker of viremia [34,35]. In the same way and considering that the genetic structure and codified viral products are similar among the Flaviviridae virus, the inclusion of GBV-C NS5 epitopes in the peptide antigen allows the amelioration of a peptide-based test for the diagnosis of GBV-C infection.

Finally, the ELISA results obtained with the chimeric MAP constructs were compared to the results obtained when using the commercial E2 recombinant protein-based test. Diepitopic MAPs

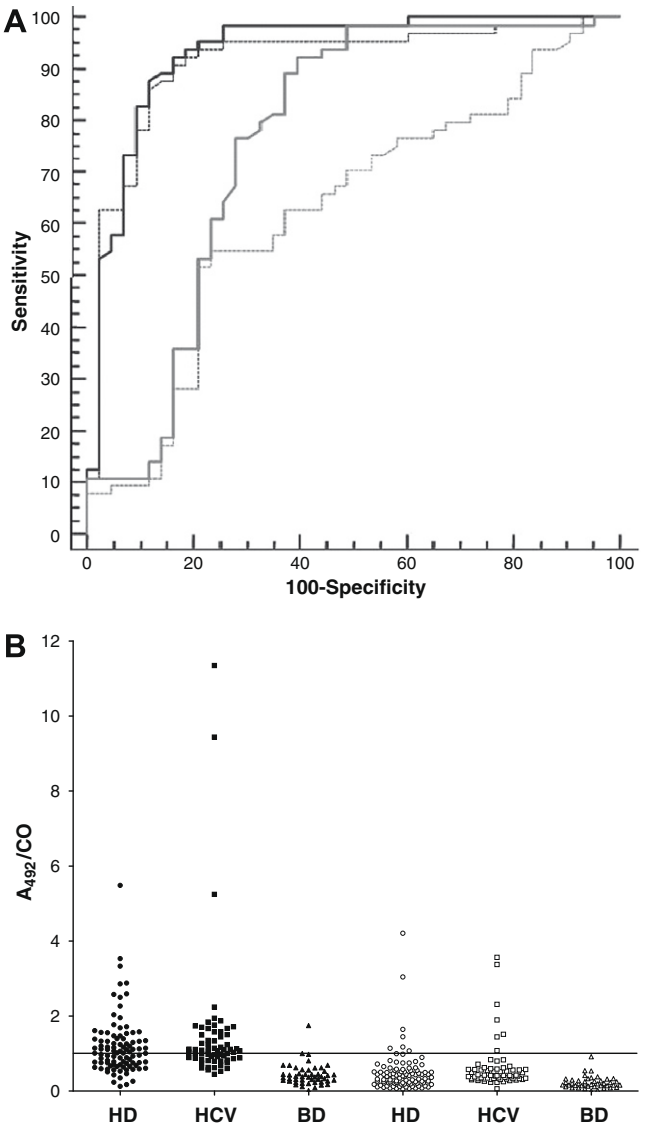

Fig. 4. (A) ROC curve analysis for MAP E2-NS5a (black) and MAP E2-NS4a (grey) antibody reactivity in the cohort of both hemodialyzed (HD, solid line) and chronic hepatitis (CH, dotted line) patient sera. Sensitivity and specificity were calculated for all potential cutoff values and plotted ROC curves. (B) Reactivity in ELISA of MAP_4(E2-NS5a) (black symbols) and MAP_4(E2-NS4) (white symbols) with HD, hemodialized patients; HCV, hepatitis C patients; and BD, blood donors. The samples were considered anti-GBV-C antibodies positive when Abs/CO $\geqslant$ 1.

were able to detect several samples that were scored as negative by the commercial E2 protein test, particularly in the chronic hepatitis panel sera (data not shown). These results reinforce the idea that the inclusion of nonstructural related sequences in the antigenic peptide increases the sensitivity of immunoassays based on E2 recombinant protein.

Detection of anti-GBV-C antibodies in human sera by surface plasmon resonance assay

Surface plasmon resonance (SPR) technology represents a promising alternative to current routine immunoassay-based methods. Due to the fact that the interaction measurements between the biomolecules are based on the changes in the refractive index on the surface of the sensor, the detection of an analyte takes place directly and without the presence of markers. The analyte does not therefore require special characteristics (scattering bands) or markers (radioactive or fluorescent) and can be detected directly without the need to use detection protocols in which multiple steps are used, for example, sandwich-type tests. However, the sensitivity, reproducibility, and robustness of SPR-based protein detection compared to the established and commonly used sandwich-based or other labeling-based methods remain to be critically assessed [36]. Comparative assessment of methodology can be complicated for complex media such as body fluids [37].

Nevertheless, the high analytical performance and the potential clinical value of this technology in the diagnosis of several infectious diseases have been reported. Thus, peptide-based biosensor assays have been performed directly on crude plasma samples for detection of specific antibodies as biomarkers of viral infectious diseases such as hepatitis A virus [38], hepatitis G virus [21], herpes simplex virus [39], rubella virus [40], and recently, Epstein-Barr virus [41] as well as other human diseases as type I diabetes [42] and cancer [43].

In this work, we have also investigated the application of a commercial biosensor instrument for the detection of specific anti-GBV-C antibodies in human serum samples. First, we tested by means of SPR technology, monomeric linear NS4b(8–22), NS5a(112–126), and two chimeric linear peptides Qm1 [NS5a(112–126)-GGG-NS4b(8–22)] and Qm2 [NS4b(8–22)-GGG-NS5a(112–126)] whose antigenicities have been previously analyzed by classical solid-phase immunoassay [20]. The chimeric peptides contained the same antigens colinearly linked via a triglycyl spacer but with two different epitope orientations. These four linear peptides were covalently linked to the CM5 sensor chip surface in order to ascertain their ability to detect anti-GBV-C antibodies in a panel of 35 hemodialyzed patient sera. The amine-coupling chemistry was used as the method of ligand immobilization in order to reproduce the same conditions of antigen presentation as in the ELISA format. Moreover a serum dilution of 1/50 was used as it has been assayed by the ELISA test.

Since the major disadvantage of SPR is the nonspecific binding of sample components to the sensor surface when analyzing complex samples such as blood serum, carboxymethyl dextran has been added to the serum samples in order to reduce the nonspecific binding to CM-coated surfaces via a competition effect. Moreover, a reference channel was also used to allow for subtraction of the signal from nonspecifically bound molecules from the total response. The reference channel was obtained after activation and deactivation of the surface without coupling a protein.

Fig. 2 (supplementary material) summarizes the serological results obtained with the different peptides using SPR methodology.

Compared to serological results previously obtained by the peptide-based immunoassays [20], the reactivity observed with the Qm1 peptide was significantly higher using SPR technology. By means of the Biacore assay the sensitivity was increased to 65% with 23 of the 35 tested sera being positive, while only 14 of 35 hemodialyzed sera samples were scored as positive in the ELISA test (sensitivity of 40%). These differences were significant ($P = 0.013$). Nevertheless, no significant differences were obtained when NS4b(8–22), NS5a(112–126), and Qm2 were immobilized in the sensor chip compared to the sensitivity previously obtained in the ELISA test (Table 2).

Taking into account that, on one hand, chimeric peptides can improve the sensitivity of the peptide biosensor assay and, on the other hand, the epitope presentation within the peptide antigen is crucial to the specific antibody recognition, we decided to analyze the other chimeric format based on a branched scaffold.

Since this chimeric branched molecule presented a good sensitivity/specificity balance in the ELISA test based on the covalent immobilization of the peptide via its amino groups, we performed the immobilization of the MAP in the sensor chip via the same amino-coupling chemistry.

Table 2
Comparison in sensitivity between SPR and ELISA assays using a panel of 35 sera from hemodialyzed patients.

	NS4b(8–22)	NS5a(112–126)	Qm1	Qm2
ELISA	8/35 (23%)	11/35 (31%)	14/35 (40%)	16/35 (46%)
SPR assay	9/35 (25%)	14/35 (11%)	23/35 (65%)	12/35 (34%)
ELISA vs. SPR	$P = 0.455$	$P = 0.053$	$P = 0.013$	$P = 0.298$

In order to allow a better discrimination of specific antibody binding from low-avidity background reactivity present frequently in human sera we optimized different conditions in the SPR analysis. First, we varied the injection time of the same concentration of the MAP peptide to obtain different immobilization levels on the three flow cells. Then, for these different peptide immobilization levels, we measured antibody binding responses at 1/50, 1/100, and 1/200 sera dilutions (Fig. 3, supplementary material).

Using these conditions, 25 sera from chronic C hepatitis patients that were considered as positive on the basis of the preliminary screening by ELISA and 11 sera from blood donors were tested. Binding of the serum antibodies was detected according to the magnitude of SPR signal before and after injection of the sample. No differences were obtained between positive and negative samples at 1/100 and 1/200 dilutions when injected over 0.034, 0.091, and 0.24 pmol mm^{-2} peptide-functionalized surfaces. Despite using reference channels and adding CM dextran to the serum samples to avoid nonspecific interaction, many serum samples behaved in an inconsistent way. Only considering the flow cell with the highest amount of peptide immobilized we could discriminate between positive and negative samples at the highest concentration of sera (1/50). However, 14 of the 25 positive samples tested gave positive results for the presence of specific serum antibodies and 5 of the 11 negative samples also a rendered positive response. Thus, the specificity of the MAP-based biosensor was significantly lower than that obtained by the conventional immunoassay.

It has been described that in immune reaction based on SPR, the specificity of the assay is enhanced by a sandwich technique, because the amplified antibody can mostly interact with its antigen [44]. The advantage of this approach does not change the physical, chemical, or biological characteristics of the antibodies. Moreover, since polyclonal antibodies contain the entire antigen-specific antibody population, one antigen molecule can form a complex with several antibody molecules. In this sense, in order to enhance the specificity of the MAP-based biosensor a sandwich strategy could be performed since the specificity of the antibody–antigen–antibody will presumably be higher than that of the antigen–antibody.

Conclusions

In our hands, nonspecific binding to the sensor surface, which would hinder a specific biological interaction, is a serious obstacle for the development of the MAP-based biosensor handling serum samples. In this sense, the MAP SPR-based biosensor was not considered as a useful tool for detecting the presence of specific serum antibodies against GBV-C since it did not give any advantage over the peptide-based ELISA. Our results demonstrated that the immunoenzymatic assays, such as peptide-based ELISA, constitute a simple, cheap, and sensitive method for detection of specific antibodies. This assay is easy to perform, cost effective, and reliable without compromising sensitivity and specificity.

The results of the immunoassays reported here highlight the usefulness of synthetic tetrameric branched peptides containing sequences from envelope and nonstructural GBV-C proteins in hepatitis G diagnosis. The potential clinical value of MAP_4(E2-NS5a) for the serodiagnosis of GBV-C infection has been demonstrated, thus giving information for performing prevalence studies of the infection among the hemodialyzed and HCV-infected population. In this sense, we are also currently investigating the ability of the chimeric MAP to recognize specific anti-GBV-C antibodies in HIV and HCV/HIV-coinfected patients in order to provide an understanding of the effect of exposure to GBV-C in the progression of illness caused by HIV infection as well as the putative role as a prognostic marker in the context of other viral infections.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2009.09.011.

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