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Detection of anti-hepatitis C virus antibodies by ELISA using synthetic peptides

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A novel ELISA assay for the detection of anti-hepatitis C virus antibodies in the sera of infected individuals is described. This assay is based on a mixture of three 15-amino acid synthetic peptides encompassing regions of core and NS4 proteins of hepatitis C virus. Comparison with other available ELISA assays based on recombinant polypeptides shows that, short synthetic peptides have the advantage over some larger recombinant peptides by giving higher specificity without loss of sensitivity.

Key words: Hepatitis C; ELISA assay; Anti-HCV antibodies; Synthetic peptides

Hepatitis C virus (HCV) is the major, if not the only cause of non-A, non-B hepatitis (1). This infection frequently progresses to chronicity (2) with a high proportion of cases evolving to cirrhosis (3,4). In the Western world HCV is the leading cause of chronic hepatitis (2). Since the isolation and cloning of HCV by Choo et al. (5), many regions of its genome have been sequenced (6–12). This has shown that the HCV is an RNA virus with a genome of 9416 base pairs coding for a polyprotein of 3010 amino acids. This polyprotein is processed by at least two proteases (one from the virus and one from the host) which free several proteins. These proteins, by analogy with those of flaviviruses and pestiviruses have been classified into structural (core, envelope or E1, NS1/E2) and non-structural (NS2, NS3, NS4 and NS5) proteins.

Kuo et al. (13) developed an ELISA assay based on a recombinant peptide (c-100) for the detection of anti-HCV antibodies in the sera of infected individuals. This assay (referred to as O1) was superceded by a second generation assay, also commercialized by ORTHO Diagnostics, based on recombinant fragments from structural and non structural regions of HCV (from now on O2). It has been reported that O1 detects anti-HCV antibodies late after infection (14) and also, may give false-positive results (15–19). In an attempt to solve these problems we developed an ELISA assay based on synthetic peptides (20). In this study the results of our assay are compared with those of O1, O2 and with a prototype developed by Welcome laboratories (from now on W) also based on recombinant antigens from structural and non structural regions of HCV.

Materials and Methods

Sera from patients and controls

Blood donors sera (panel 1): The sera from 221 blood donors were used as negative controls.

Chronic non-A, non-B hepatitis sera (panel 2): The sera from 150 patients fullfilling the following criteria: liver biopsy showing chronic persistent or chronic active hepatitis with or without cirrhosis, presence of raised serum alanine aminotransferase for more than 6

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months, negativity for HBsAg, alcohol consumption less than 50 g/day, antinuclear antibodies negative or a titre lower than 1/100, absence of potential hepatotoxic drugs, normal levels of ceruplasmin and α-1-antitrypsin.

Chronic hepatitis B sera (panel 3): The sera from 98 HBsAg positive patients with biopsy proven chronic hepatitis.

Other sera (panel 4): 115 serum samples consecutively received in the laboratory from hemodyalisis patients and patients with hypertransaminasemia.

Peptide synthesis

Peptides were synthesized by the solid-phase method of Merrifield (21) using the Fmoc alternative (22) with a manual multiple solid-phase synthesizer which can synthesize up to 96 independent peptides (23). The ninhydrin test of Kaiser (24) was used to monitor the coupling and deprotection steps. At the end of the synthesis, peptides were cleaved and deprotected followed by two washings with diethyl ether. They were used for the ELISA assays without further purification.

ELISA assay

The ELISA plates (Nunc-Immunoplate MaxiSorp, Denmark) were coated at 4°C overnight with a mixture of peptides dissolved in 0.1 M sodium carbonate. This was done by adding 200 μl/well of this solution containing 5 μg/ml of each peptide. The plates were washed three times with phosfate-saline buffer (pH 7.2) containing 0.1% of Tween-20 (PBST). The plates were then incubated at 37°C for 45 min with 210 μl/well of a 1/21-serum dilution in PBST containing 1% powdered milk (PBSTM). Two positive and three negative sera were used as controls. After washing three times with PBST, 200 μl/well of 1/2000 biotinilated goat anti-human antibody (Amersham, UK) and 1/500 streptavidine peroxidase (Amersham, UK) in PBST was added and incubated at 37°C for 30 min. After 3 washings with PBST, the plates were developed in the dark at room temperature for 15 min with 100 μl/well of a solution of 0.4 mg/ml of *o*-phenylendiamine in 0.05 M citrate (pH 5) buffer containing 0.014% hydrogen peroxide. The reaction was stopped by adding 75 μl/well of 4 N sulphuric acid. The plates were read at 492 nm using Titertek Multiscan MK II. The cut off was calculated by adding 0.4 optical density units to the average reading of the three negative control sera.

Commercial ORTHO HCV ELISA assays

These assays (Ortho first and second generation assays, O1 and O2) were carried out according to the manufacturers (Ortho Diagnostics Systems, Raritan, New Jersey) instructions.

Wellcome HCV assay

This assay is an ELISA assay based on a recombinant viral antigen polypeptide named BHC10 encompassing regions from both the core and NS5 regions of the virus (Wellcome Diagnostics, Beckenham, Kent). It was carried out as described elsewhere (25).

RIBA assay

The second generation RIBA assay from CHIRON was carried out according to the manufacturers instructions.

Serological markers of hepatitis B virus infection

Serological markers of hepatitis B viral infection were investigated by RIA or ELISA with commercial reagents (Ausria II, Ausab, Corab, IMx HBsAg, Ausab EIA, IMx core; Abbott Laboratories, Chicago, Illinois).

PCR analysis

This analysis was carried out after reverse transcription of non-A, non-B viral sequences to cDNA. The amplification was done as described (25) using primers from the 5′ non-coding region of the virus (26).

Results

In order to select linear epitopes useful for ELISA from the proteins coded by the HCV genome, over 300 peptides of 15 amino acids in length were synthesized. These peptides encompass most regions of viral proteins. The core and envelope proteins were especially explored with three sets of peptides to cover the whole sequence and each set differing from the other by a frame shift of 5 amino acids.

To detect the peptides that were recognized by the majority of non-A, non-B sera and to discard those that were recognized by the immunoglobulins other than anti-HCV immunoglobulins, all peptides were tested by ELISA against the sera of 31 blood donors and 65 patients with non-A, non-B chronic hepatitis.

It was found that three peptides (two from core and one from NS4 proteins) taken together would allow the detection of anti-HCV antibodies in 95% of the non-A, non-B sera without cross-reacting with any of the healthy control sera. These results encouraged us to use a mixture of these three peptides to develop an ELISA

assay for the detection of anti-HCV antibodies in the sera. From now on this assay will be referred to as P. To compare the specificity and sensitivity of O1, O2, W and P assays, the sera were classified as follows:

HCV positive serum: The serum is positive if the result is positive for at least one pair of the following assays: O2, W, P, RIBA, PCR.

HCV negative serum: The serum is negative if the result is negative for all assays or positive for only one of them.

These definitions were used to classify the sera from four different panels.

All the sera from panel 1 (221 blood donors) were classified as negative because they were negative to O1, O2, W and P assays.

The results obtained with panel 2 (non-A, non-B chronic hepatitis patients) are shown on Table 1a. The sera were classified as 142 positive and 8 negative. The 142 positive sera were detected by O2, W and P but only 108 were detected by O1. Nineteen sera, from the 34 not detected by O1 but detected by O2, W and P, were tested by PCR. Eighteen out of the 19 (94.7%) sera tested were PCR positive.

The results obtained with panel 3 (hepatitis B patients) are shown on Table 1b and Fig. 1. The sera were classified as 22 positive and 76 negative. Assays O1, O2, W and P gave 13, 13, 0 and 1 false-positive results, respectively because they attributed a positive result to a serum classified as negative. Similarly, O1, O2, W and P gave 9, 1, 5 and 3 false-negative results.

The results obtained with panel 4 (other sera) are shown on Table 1c. The sera were classified as 56 positive and 59 negative. Assays O2, W and P gave 2, 0 and 1 false-positive and 2, 4 and 3 false-negative, respectively.

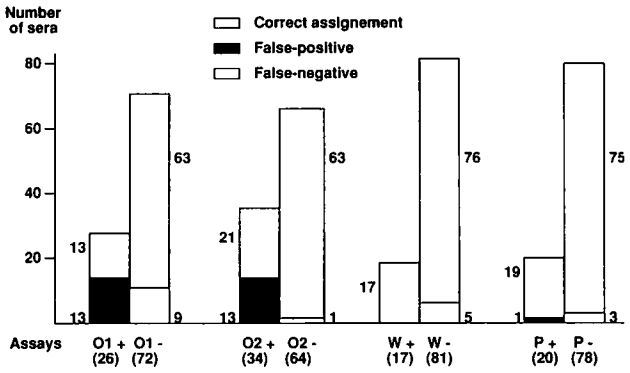

Fig. 1. Comparison of the efficacy of 4 ELISA assays: O1, O2, W and P in the detection of anti-HCV antibodies in the sera of 98 patients with chronic hepatitis B (panel 3). The number of positive and negative results obtained with each assay are indicated in brackets under each bar.

Taking into account the results obtained with the four panels of sera (584 sera in total) we calculated the percentages of sensitivity and specificity of O2, W and P. These percentages are shown in Table 2.

TABLE 1

Positive results assigned by O1, O2, W and P ELISA assays in three panels of sera. Concordance of these assignations with the final classification[a] of these sera

Classification	Number of positive sera to			
	O1	O2	W	P
(a) Panel 2: non-A, non-B hepatitis sera				
142 HCV positive sera	108	142	142	142
8 HCV negative sera	0	0	0	0
(b) Panel 3: Hepatitis B sera				
22 HCV positive sera	13	21	17	19
76 HCV negative sera	13[b]	13[b]	0	1
(c) Panel 4: other sera				
56 HCV positive sera	NT	54	52	53
59 HCV negative sera	NT	2	0	1

[a]The definitions for positive and negative anti-HCV sera are given in Results section.
[b]Seven of these 13 sera are the same in O1 and O2 assays (NT) not tested.

Discussion

The results for non-A, non-B sera (Table 1a) show that assays O2, W and P have the same sensitivity. This sensitivity is much higher than O1. Clearly, the extra recombinant peptides added to O1 to give O2 have increased assay sensitivity by providing additional 'recognizable' epitopes.

With respect to assay specificity the results of Table 1 and Fig. 1 indicate that both W and P are more specific than O1 and O2. Moreover, the similar lack of specificity of O1 and O2 found in panel 3 (false-positives shown in Fig. 1 and Table 1b) might be due to a cross-

TABLE 2

Comparison of the sensitivity and specificity of O2, W and P ELISA assays

	Assays		
	O2	W	P
Sensitivity	98.6%	95.9%	97.3%
Specificity	95.9%	100%	99.4%

reaction of antigen c-100 (which is used in O1 and O2) with antibodies other than anti-HCV antibodies. This suggests that a similar epitope might be present in the proteins of hepatitis B virus and the c-100 antigen. To explore this hypothesis a sequence homology between c-100 and the different proteins from HBV was looked for. The peptide LQTLPANPPPAS, amino acids 75–87 of pre-S1 protein of ayw HBV serotype (one of the two most common serotypes in Spain) were found to be very similar to the peptide LSTLPGNPAIAS, amino acids 1778–1789 from the NS4 region of HCV and contained by c-100 antigen. Indeed, eight out of 12 amino acids are identical in both peptides. These two peptides were synthesized and used as antigens in an ELISA assay against the false-positive of O1 and O2 of panel 3. Since it was found that 42% of these sera recognized both peptides, we believe that these antibodies might be responsable for some of the false positive results observed when using the O1 and O2 assays.

An ELISA assay based on synthetic peptides has been recently published by Hosein et al. (27). We have not had access to this assay and for this reason were unable to compare it with our own. Moreover, since their results are only compared with O1, which has low sensitivity and poor specificity, no conclusions could be drawn concerning its relative value with respect to O2, W and P assays.

Because of their size, recombinant protein fragments have the advantage of containing a potentially high number of 'recognizable' epitopes. For similar reasons they also have the risk of encompassing epitopes that might cross-react with other antibodies not elicited by the HCV and give false-positive results — antibodies induced by a common antigen like those from influenza virus for example — . Moreover, recombinant peptides are usually expressed as fusion peptides with a protein that is easily expressed. This enhances the possibility of cross-reactivity even more. Thus, it has been reported (16) that superoxidismutase (SOD, the fusion protein with which the c-100 fragment of the O1 assay is expressed) might be responsible for the false-positive results of O1 in the case of autoimmune chronic active hepatitis.

Unlike recombinant polypeptides, short synthetic peptides (of around 15 amino acids) cannot detect antibodies induced against most conformational epitopes of the antigen. However, since they restrict antibody recognition to smaller molecules, the risk of possible cross-reaction with undesirable antibodies is diminished. Indeed, as shown in Table 2, anti-HCV antibodies can be detected by ELISA with short synthetic peptides with similar sensitivity and better specificity than with some large recombinant antigens.

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