THE CORE-DIRECTED IMMUNE RESPONSE IN HEPATITIS C VIRUS INFECTION REVEALS A DISCONTINUOUS EPITOPE PRESENTED BY THE CORE N-TERMINAL REGION.

ABSTRACT

Hepatitis C virus (HCV) infection exhibits a specific antibody response against all HCV antigens, which could play a role in disease control. In order to gain insight into these matters we generated a panel of monoclonal antibodies (MAB) against a synthetic peptide resembling the first 28 aa (aa) of the core protein, and used it to analyze synthetic peptide libraries of the same region. By this means, three different MABs identified two separate sequences, NPKPQR (aa 4-9) and RNTNRR (aa 13-18), suggesting that these antibodies are directed against a discontinuous core epitope. In order to state if the folding of the core protein would result in the same epitope, we used a recombinant HCV core protein, expressed in Pichia pastoris. This allowed us to show that this epitope is present in the protein. The results of this work suggest a possible escape mechanism of virus from the immune system.
INTRODUCTION

Hepatitis C virus (HCV) is the major etiological agent of post-transfusion and parenterally transmitted, sporadic non-A, non-B hepatitis throughout the world (Alter et al. 1989; Bukh et al. 1994). About half of all patients with acute hepatitis C progress to chronic disease (Farchi et al. 1991) and many of them eventually develop hepatocellular carcinoma (Wang et al. 1996). Therefore, interest has focused upon the function of viral proteins and their interactions with the immune system during the course of infection.

In contrast to the substantial variation of envelope glycoproteins, E1 and E2, the core protein is well conserved among different HCV genotypes (Bukh et al. 1994) and anti-core antibodies are present in the majority of patients with chronic infection (Harada et al. 1991, Manzini et al. 1993). As an example, the recombinant C12-3, amino acids (aa) 2-120 of core protein, is the major component of the commercially available second-generation anti-HCV tests (Chemello et al. 1993, Holsein et al. 1991).

Besides, HCV core protein contains immunodominant epitopes that elicit antibody production at an early stage of HCV infection (Harada et al. 1991). Also, a highly antigenic site in HCV core recognized by both murine and human cytotoxic T lymphocytes (CTL) was identified (Wang et al. 1996). This and other studies suggest that animal model experiments using mice to identify the antigenic epitopes in HCV may be useful for the identification of these epitopes in humans (Kakimi et al. 1995).

The N-terminal portion of the HCV core protein contains a major antigenic region for B cells (Holsein et al. 1991). Murine sera from two different strains of mice immunized with the hydrophilic portion of HCV core protein (aa 1-115) bound to the N-terminal portion of the core protein at aa 1-28 and demonstrate that murine and human B cells can recognize the same epitope of HCV core protein (12). More recently, using the phage display system for peptide library identification of epitopes, a new human B cell epitope was defined within the immunodominant N-terminal region of HCV core protein (aa 8-14) (Barban et al. 2000).

In order to gain insight into the antigenic characterization of this area of the N-terminal domain of HCV core protein, we generated monoclonal antibodies (MABs) against a synthetic peptide spanning core aa 1 to 28 and screened synthetic peptide libraries with these MABs. We also used recombinant core protein to study the presence of this epitope in HCV core protein. The results of this study suggest the presence of a new discontinuous epitope present within the immunodominant N-terminal region of the HCV core protein.

MATERIAL AND METHODS

Mice.

BALB/c mice were obtained from the breeding colony at Instituto de Higiene (Uruguay). Female mice, 40 days of age at the initiation of the experiments, were used in all studies.
Peptide synthesis.
We manually synthesized a peptide spanning the first 28 aa of the N-terminal domain of core protein by the Fmoc procedure (Frank & Doring, 1988). The peptide sequence was:
M-S-T-N-P-K-P-Q-R-K-T-K-R-N-T-N-R-R-P-Q-D-V-K-F-P-G-G-G. This sequence is a consensus sequence for almost all HCV strains, from all types and sub-types, isolated to present in different geographic regions of the world. The peptide was purified by High Performance Liquid Chromatography (HPLC) to 90 % purity and lyophilized until use.

Immunization of mice with synthetic peptide.
Mice were immunized with 10 μg synthetic peptide on days 0, 15, 45, 75 and 105 in equal volume of Freund’s complete adjuvant (FCA) (Difco) for the first injection and Freund’s incomplete adjuvant (FIA) for the rest of them, by subcutaneous injection into the dorsal regions. Control mice were immunized with FCA alone.

Monoclonal antibodies.
Mice splenocytes were fused with murine myeloma cells obtained from The Scottish Antibody Production Unit (SAPU), as previously described (Kohler & Milstein, 1975). After fusion cells were plated in five 96-well plates, in HAT media, for selection of hybridomas and incubated in a CO₂ incubator for 10 days. After incubation, 100 μl of supernatant from each well was assayed by ELISA for the detection of antibody producing hybridomes. Those tested positive were expanded, first to a 24 well plate and later to bottles of 25 cm² and 75 cm².

All hybridoma cell lines considered of interest were frozen at –80°C until use.

Monoclonal antibody purification.
Supernatants from suitable cell lines were purified using a column of Protein A Sepharose (from Sigma) and HPLC.

Immunoglobulin characterization.
In order to determine the sub-type of immunoglobulin of the monoclonal antibodies obtained, we used a BIO-RAD kit according to instructions from the manufacturers.

Peptide libraries.

Monoclonal antibodies have been incubated separately with peptide libraries for 1 hour at room temperature using the following concentrations: E8: 8.75 μg/ml, C10: 12 μg/ml, H2: 6.8 μg/ml and A5: 19 μg/ml in MBS (Membrane Blocking Solution. N° SU-07-250; Genosys Biotechnologies, Cambridge
was developed for 15 minutes with TMB as substrate (obtained from Sigma) and stopped with 2M sulphuric acid. After reading, results were expressed as optical density (OD<sub>450 nm</sub>).

**RESULTS**

A total of 4 MABs were obtained against the synthetic peptide resembling the first 28 aa of HCV core protein (C10, H2, E8 and A5). All MABs had isotype IgG1.

To identify more precisely the recognition sites of these antibodies, we used them to analyze synthetic peptide libraries of the same region of the core protein. The results of this study is shown in Fig. 1. As it can be seen in the figure, all MABs recognized spot 13 (RNTNRR, aa 13-18). Other spots are also recognized by the MABs. Interesting, a clear reactivity was observed with spot 4 (NPKPQR, aa 4-9) with three different MABs, showing that a discontinuous epitope may be present in that region of the HCV core protein (see Fig. 1).

Then, in order to study the determinant aa involved in immune recognition of this epitope, we changed aa at the amino- and carboxy-terminal ends of the epitope. By these means, it was possible for us to identify N4 as the principal determinant of the NPKPQR amino-terminal end of the epitope and aa N16, R17 and R18 as determinants for RNTNRR carboxy-terminal end of the epitope (not shown).

In order to study if our monoclonal antibodies recognize the appropriate, complete viral antigen
(e.g. the core protein itself) and not just short peptides, MABs C10 and H2 (which recognize the discontinue epitope, see Fig. 1) were tested in an ELISA assay using HCV core protein, expressed in Pichia pastoris, as an antigen by coating it to the surface of the plate. The results of this study are shown in Fig. 2. As it can be seen in the figure, there is a proportional relationship among antibody concentration and optical density. This speaks for a specific recognition of HCV core protein by both antibodies and that the epitope we have already described is indeed present in the protein (see Fig. 2).

A spatial model of 4 HCV core protein molecules have been obtained by nuclear magnetic resonance (Ladavie et al., 1999). We have identified in this three dimensional structure model the aa sequences involved in the proposed discontinuous epitope. This is shown in figure 3.

DISCUSSION

The N-terminal region of the capsid protein is highly hydrophilic and contains multiple overlapping epitopes. Studies in the murine system as well as in humans suggests that there is at least one major conformational epitope residing within the first 82 aa of the HCV core protein (Cerino et al. 1991, Maradpour et al.1996). Other studies have shown the presence of a linear antigenic determinant, located mainly between residues 9 to 16, recognized by nearly 100% of patients (Wang et al. 1996). Nevertheless, the reason why these are not neutralizing antibodies for the infection remains unknown.

Three different MABs (E8, C10 and H2) recognize two separate areas on the first 28 aa of HCV core protein (see spot 4 and 13, Fig. 1), suggesting the presence of a discontinuous epitope on the N-terminal region of HCV core protein (see Fig. 1). These results can not be due to unspecific MABs binding, since the experiments were repeated several times with different peptide libraries, showing the specificity of the aa sequences involved in MABs recognition. Besides, experiments done with recombinant HCV core protein, expressed in Pichia pastoris, showed that these MABs specifically bind to the recombinant core protein (see Fig. 2) showing that this epitope is present in the viral antigen (e.g. the core protein itself).

These results are also in agreement with three-dimensional structural analysis of HCV core protein in which the spatial disposition of the first 28 core aa makes possible the existence of a discontinuous epitope in that area (Perin et al. 1997).

Recent structural analyses and molecular modeling of four core protein molecules allowed the characterization of a three-dimensional motif composed by two alpha-helix (R17-F24 and Q29-L37) separated by a loop (P25-G28) (Jovelit-Reynaud et al. 998). As it can be seen in Fig. 3, spot 13, highly recognizable by all MABs assayed (see Fig. 1) is situated in a region where the model coincide for the four molecules (see Fig. 3, region B); while spot 4, recognized by MABs E8, C10 and H2 (see Fig. 1) is situated in a region
that allows the protein to have a flexible conformation (see Fig. 3, region A), so different modeling results are obtained for each of the four molecules (Fig. 3, region A).

These results suggest that the flexible conformation of the N-terminal region of the core protein might permit different spatial conformations be presented to the immune system, involving a possible escape mechanism of virus from immune surveillance. This may also contribute to explain the presence of non-neutralizing antibodies directed to this highly conserved region in nearly 100% of HCV chronic infected patients.

Other discontinuous epitopes have been recently reported for other HCV proteins, like the hypervariable region 1 of E2 (Roccasceca et al. 2001) or the NS3 protein (Zang et al. 2000). This speaks for the need of a detailed immunological characterization of all HCV proteins in order to state the possible escape mechanisms of HCV from immune surveillance.

REFERENCES


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Figure 1. **Spotscan of membrane-bound N-terminal HCV core peptides with monoclonal antibodies.** Each peptide sequence was assayed with 4 different monoclonal antibodies, whose names are shown on the left side of the figure. Each peptide (6 mers) overlap in one amino acid with the following peptide. A diagram is shown with the results obtained for each monoclonal antibody. Numbers in the diagram show spot number. Spots are represented by ovals. Spot strongly recognized 2 minutes after the developer was added are shown by a dark gray oval with thick black borders. Other spots recognized by the monoclonal antibodies are shown by a dark gray oval with thin borders. Spots not recognized are shown by light gray ovals with thin borders.

Figure 2. **Specific binding of MABs to recombinant HCV core protein in ELISA assays.** Results of an ELISA assay done with MABs C10, and H2 is shown. Numbers at the bottom of the figure shows MABs serial dilutions (1:10, 1:5, 1:2 means 1 into 10, 1 into 5 and 1 into 2 dilutions; 1:1 means undiluted). Numbers on the left side of the figure denotes optical density (OD$_{450}$).

Figure 3. **Spatial model of aa 2-45 of four molecules of Hepatitis C virus C protein.** Spot 4 from peptide library is shown by A and the major epitope determinant sequence is shown by B (both in white). C shows the first alpha helix and the loop is indicated by letter D, both in light gray. The region E corresponds with aa 29-45 and is indicated by dark gray.