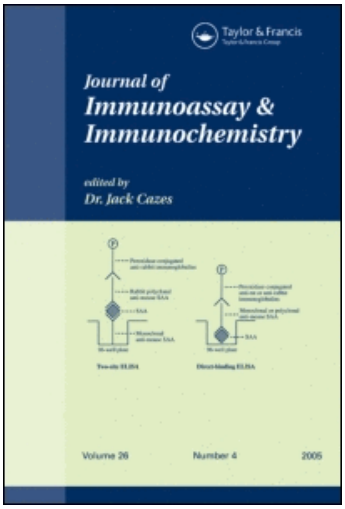

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[a] Department of Biomedical Technology, National Research Center, Cairo, Egypt [b] Naval American Medical Research Unit # 3, Cairo, Egypt [c] Department of Tropical Medicines, Cairo University, Egypt [d] National Liver Institute, Menoufiya University, Shibin El Kom, Egypt [e] Biotechnology Research Center, New Damiettea City, Egypt

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Establishment of Hybrid Cell Lines Producing Monoclonal Antibodies to a Synthetic Peptide from the E1 Region of the Hepatitis C Virus

Ashraf A. Tabll,[1] Samy B. Khalil,[2] Reem M. El-Shenawy,[1] Gamal Esmat,[3] Amr Helmy,[4] Abdel Fattah Attallah,[5] and Mostafa K. El-Awady[1]

[1]Department of Biomedical Technology, National Research Center, Cairo, Egypt
[2]Naval American Medical Research Unit # 3, Cairo, Egypt
[3]Department of Tropical Medicines, Cairo University, Egypt
[4]National Liver Institute, Menoufiya University, Shibin El Kom, Egypt
[5]Biotechnology Research Center, New Damiettea City, Egypt

Abstract: We aimed at establishing hybridoma cells secreting monoclonal antibodies (mAbs) against E1 synthetic peptide of HCV. BALB/c mice were immunized with HCV E1-synthetic peptide (GHRMAWDMM) and its spleenocytes were fused with the P3NS1 myeloma cell line. Two highly reactive and specific mAbs (10C7 IgG2b mAb, and 10B2 IgG1 mAb) were generated. The target HCV E1 antigen was identified at ~38 kDa in serum of infected individuals. A newly developed ELISA detected the target antigen in 90% of sera from HCV RNA infected individuals with a specificity of 84%. So, the generated mAbs may provide promising probes for serodiagnosis of HCV infection.

Keywords: Hepatitis C virus, Hybridoma, Monoclonal antibody, Envelope 1, Antigen, Diagnosis

Correspondence: Dr. Ashraf A. Tabll, Department of Biomedical Technology, National Research Center, Dokki, El-Behoose St. P. O. Box 12622, Giza, Cairo, Egypt. E-mail: ashraftabll@yahoo.com

INTRODUCTION

Hepatitis C virus (HCV) is a leading cause of chronic liver disease, cirrhosis, and hepatocellular carcinoma, as well as being the most common indication for liver transplantation in many countries. Although the incidence of HCV infection has dramatically decreased during the past decade, the worldwide reservoir of chronically infected persons is estimated at 170 million, or 3% of the global population.[1] In Egypt, HCV infection is a major health problem.[2–5] Recently, we developed a rabbit polyclonal antibody to E1 region and used it in blocking binding and entry of HCV virion infection into target cells,[6] for detection of HCV E1 antigen on blood lymphocytes,[7] and also on an infected HepG2 cell line.[8] In addition, this antibody gave satisfactory results for detection of circulating HCV E1 antigen in serum samples from infected HCV patients.[9]

In this study, we aimed to develop hybridoma cells secreting monoclonal antibodies (mAbs) against HCV E1 synthetic peptide and to investigate its properties and to use these antibodies for detection of HCV E1 in serum samples from individuals infected with HCV.

EXPERIMENTAL

Antigen used for Immunization

Synthetic peptide of HCV E1 region was used for immunization. The peptide was synthesized commercially (Biosynthesis Inc., Lewisville, Texas, USA); its sequence was GHRMAWDMM. The peptide was synthesized in the amide form using standard solid phase synthesis involving 9-flurenylmethoxy carbonyl chemistry and was purified using HPLC as previously described.[6]

Animal Immunization

Eight-week-old BALB/c mice were immunized by subcutaneous injection of 10 μg/mouse of HCV E1 synthetic peptide conjugated with BSA. The antigen was prepared in phosphate-buffered saline (PBS, pH 7.2) and mixed with equal volumes of Freund's complete adjuvant (Sigma, St. Louis, MO). The second and third injections were performed in three weekly intervals by using Freund's incomplete adjuvant. Four days before fusion, a booster injection of 10 μg of antigen in 0.1 mL of PBS without adjuvant was performed intravenously. The ELISA and dot-EIA (described later) were used for screening the reactivity of immunized mice sera.

Parental and Cell Line used for Fusion

The lymphocytes from the spleen of the BALB/c mouse immunized with E1 synthetic peptide conjugated with BSA (mouse sera gave high OD by ELISA) and mouse myeloma cells (P3NS1) were used for fusion.[10] The myeloma cells were cultivated in DMEM medium (Gibco-Scotland, Paisley, UK) supplemented with 20% fetal calf serum under a humidified atmosphere of 5% CO_2. For hybridoma preparation, the spleen was used as lymphocyte source. Cells were resuspended in serum free DMEM media and washed three times in the same buffer. Fusion of spleen cells and myeloma cell line (P3NS1), at a ratio of 10:1, was achieved. All reagents to be used for fusion were warmed to 37°C. Spleen cells and myeloma cell were mixed, centrifuged, and resuspended in 1 mL polyethylene glycol solution (Hybri-Max®, Sigma) over 1 min, followed by serial additions of medium over 5 min, with stirring. After centrifugation, fused cells were resuspended in 1 × HAT medium (HAT Media Supplement 50×, Hybri-Max) and OPI Media Supplement (Hybri-Max®, Sigma) and plated onto microtiter plates at 100 μL/well. Cells were incubated for about 10 days until assay results were confirmed, at 37°C, with 5% CO_2. Cells that produced antibody under investigation were expanded for cloning. The expanded master cultures were frozen and stored in liquid nitrogen for future use. The HAT medium was replaced with HT (hypoxanthine, thymidine) medium (HT Media Supplement (50 ×) (Hybri-Max®) after 10 days.

Selection and Cloning Procedure

Positive hybrid clones producing antibody with the highest specificity were sub-cloned by a limiting dilution method. At each stage of growth, aliquots of hybrid cultures (3–5 million cells) were frozen in liquid nitrogen in 80% DMEM, 20% FCS, and 10% dimethylsulphoxide (Sigma). The ELISA and dot-EIA (described later) were used for screening reactivity of hybridoma supernatants. Hybrid cells producing mAb, showing the highest optical density by ELISA and highest reactivity by dot-ELISA, were selected and subjected to subsequent sub-cloning steps by a limiting dilution method.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA plates were coated with 2.5 μg/mL of HCV E1 free peptide in PBS and incubated overnight at 4°C. The plates were washed three times with PBS-T20 washing buffer and blocked with 200 μL of 0.2% BSA in PBS for 30 min at 37°C. After washing, the culture supernatant (100 μL/well) of hybridoma growth wells or immunized mice sera was added and incubated for 90 min at room temperature. Serum of non-immunized mouse was used

as negative control. The bound antibodies were detected with peroxidase conjugated goat anti-mouse IgG antibodies (Sigma) diluted 1/2,000 in PBS buffer for 90 min at room temperature. The plates were washed five times and o-phenylenediamine (OPD, 0.01%) substrate (Sigma) was added. Following color development, the immunoreactivity of mAbs (expressed in optical density units) was measured at 450 nm in a micro plate ELISA reader.

Dot-Enzyme Linked Immunosorbent Assay (Dot-ELISA)

Two μL of HCV E1 synthetic peptides conjugated with BSA with concentrations of 5 μg/mL PBS was spotted on the nitrocellulose membrane filter (Sigma). Blocking with 1% BSA in PBS was done for 30 min at RT with constant shaking. Hybridoma supernatants were added to 0.1 BSA in PBS 0.05% T20 for 90 min at RT with shaking. After washing three times with PBS-T20, the strips were incubated with goat-anti mouse IgG-HRPO conjugate diluted 1:2,000 according to the manufacturer's instructions (Jackson Immuno Research Labs Inc., USA). Following washing, substrate solution (4-chloro-1-naphtol dissolved in 96% ethanol and 0.03% H_2O_2) was added to the strips, and incubated at room temperature for 20 min. The reaction was stopped after 5 min by replacing the substrate with distilled water. Normal mouse serum and immunized mouse serum were used as negative and positive controls; respectively.

Isotyping of the Generated Monoclonal Antibodies

Heavy and light chains of developed immunoglobulins produced by the hybrid cell lines were identified using an Hbt Mouse mAb Isotyping test kit (HyCult Biotechnology b.v., USA).

Production of Ascitic Fluid in Balb/c Mice

Prime adult female mice Balb/c (6–8 weeks old) were injected with 0.5 mL of pristane (2,6,10,14-tetramethyldecanoic acid) in the peritoneal cavity. After 7–14 days, mice were injected intraperitoneally with 5×10^5 to 5×10^6 hybridoma cells in a volume of 0.1–0.5 mL. Ascitic fluid began to accumulate within 1–2 weeks following the injection of cells. When the mouse was noticeably large, we tapped the fluid, but before the mouse had difficulty moving. The ascitic fluid was collected by inserting a syringe needle (18–22 gauge) into the lower abdomen and collecting the ascites in a tube as it dripped. The ascites fluid was incubated at 37°C for 1 hr and transferred to 4°C overnight. The fluid was spun at 3,000 × g for 10 min and the supernatant was carefully removed from the cell pellet.

Serum Samples

Serum samples of 100 HCV infected Egyptian patients (aged 12–57 years; mean age of 38.35 years) from Medical Service Unit, National Research Center, Giza, Egypt, and 25 healthy volunteers (aged 15–58 years; mean age of 36.6 years) were included in the present study. Anti-HCV antibodies and HCV RNA were detected in all patients. An informed consent was obtained from all individuals participating in the present study; they were fully informed concerning the nature of the disease and the diagnostic procedures involved.

Western Blotting

Serum samples of HCV infected and non-infected individuals were subjected to sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)[11] through 4% stacking and 12% resolving gels in 0.75 mm-thick vertical slab gels. Serum samples were diluted; 1:100 in PBS, mixed with the sample buffer (0.125 M Tris base, 4% SDS, 20% glycerol, 10% mercaptoethanol, and 0.1% bromophenol blue as a tracking dye), and immediately boiled for three minutes. A mixture of reference proteins was run in parallel. Gels were then stained with Coomassie blue Western blotting was performed as follows: resolved samples separated by SDS PAGE were electro-transferred onto nitrocellulose membranes (0.45 mm pore size). Blotting was carried out at a constant voltage of 60 volts for two hours. The immunoassay was then performed using mouse monoclonal antibody anti-HCV E1 diluted 1:100 in blocking buffer with constant shaking. The blots were then washed, followed by incubation for 2 h with goat anti-mouse IgG alkaline phosphatase conjugate (Zymed) diluted 1:1,000 in Tris-buffered saline [TBS; pH 7.4]. The nitrocellulose membrane was then soaked in premixed alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate [BCIP], nitro blue tetrazolium [NBT], (Zymed). The color was observed within 10 minutes and the reaction was stopped by the addition of distilled water.

Detection of E1 Antigen in Serum using ELISA

Polystyrene micro titer ELISA plates were coated with 50 μL/well of tested serum sample diluted in carbonate/bicarbonate buffer (pH 9.6). The plates were incubated overnight at room temperature and washed three times using 0.05% (v/v) PBS-T20 (pH 7.2); then, free active sites were blocked using 0.2% (w/v) nonfat milk in carbonate/bicarbonate buffer. After washing, 50 μL/well of anti-HCV-E1 mouse monoclonal antibody, diluted 1:1,000 in PBS-T20, were added, and incubated at 37:C for 2 h. After

washing, 50 μL/well of anti-mouse IgG peroxidase conjugate, diluted in 0.2% (w/v) nonfat milk in PBS-T20, was added and the mixture was incubated at 37°C for 1 h. The amount of coupled conjugate was determined by incubation with 50 μL/well o-phenylenediamine (OPD, 0.01%) substrate (Sigma) for 30 min at 37°C. The reaction was stopped using 3M HCl and the absorbance was read at 450 nm.

RESULTS

Generation and Screening of Hybrid Cell Lines

HCV E1 synthetic peptide, mixed with Freund's adjuvant, stimulated a good response after injection into a group of mice, Figure 1. Only mouse # 1 was selected for spleen cell fusion. Of 236 growing hybrid cells, 18 mAbs supernatants exhibited moderate to strong reactivity in ELISA and against HCV E1 free peptide. Only two hybrid cells producing monoclonal antibodies (10C7 and 10B2) and showing the highest optical density by ELISA were selected and subjected to subsequent sub-cloning steps by a limiting dilution method, Figure 2.

Specificity

Hybridoma supernatant from 10C7 (clone 1 G10) and 10B2 (clone 3A6) were tested by ELISA using vaious proteins such as E1 peptide, HCV core peptide,

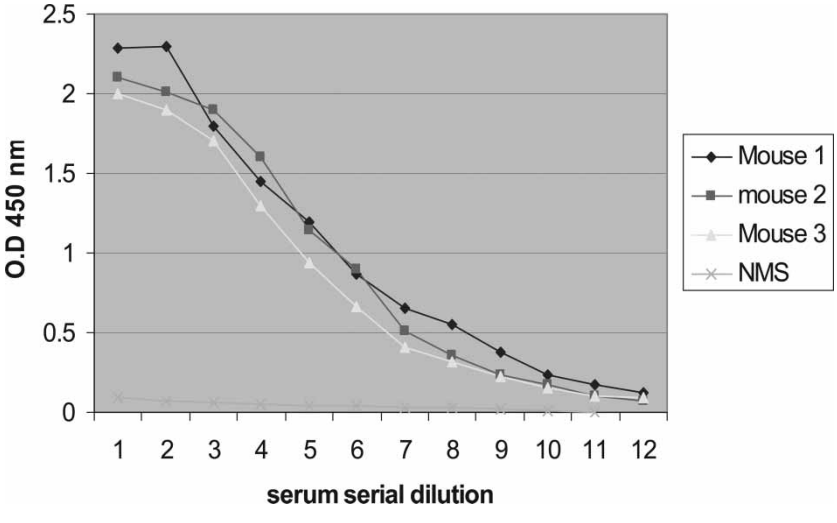

Figure 1. Screening of immunized mouse with HCV E1 peptide (2.5 μg/ml) by ELISA. Serial dilutions of immunized and normal mouse serum were used starting from dilution of 1:100 in phosphate buffer saline 0.05% T20.

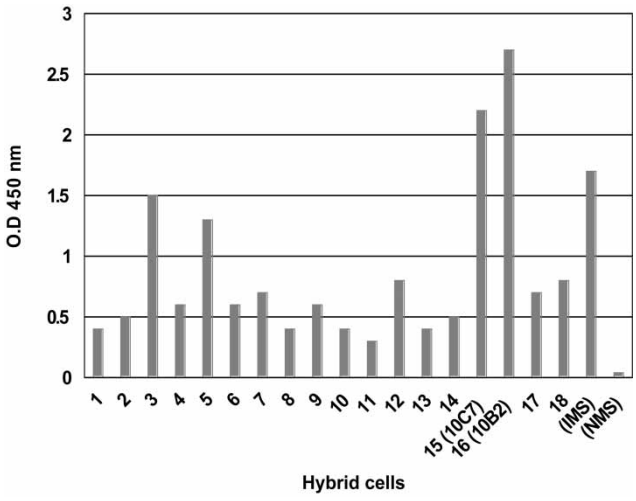

Figure 2. Screening of growing hybrid cells by ELISA. Of 236 growing hybridomas, 18 mAb supernatants exhibited moderate to strong reactivity in enzyme-linked immunosorbent assay (ELISA) and against HCV E1 free peptide (2.5 μg/ml). We select only the two strongest hybridoma cells (10C7 and 10B2). Immunized mouse serum (IMS) was used as positive control and non-immununized mouse serum (NMS) as negative control.

bovine serum albumin, and normal human serum. They did not give any cross-reaction with the related proteins. mAbs reacted with both E1 peptide alone (free) and E1 conjugated with BSA) and did not react with BSA alone, Table 1. The production of monoclonal antibodies from (10C7 clone

Table 1. Specificity and reactivity of the produced monoclonal antibodies against HCV E1 synthetic peptide and BSA, Human serum and HCV core antigen

	Monoclonal antibodies (OD)			
Protein used in ELISA	10C7 (clone 1G10)	10B2 (clone 3A6)	Positive control (OD)	Negative
E1 free	1.9	1.80	2.0	0.04
E1 conj. with BSA	1.8	1.70	2.1	0.04
BSA alone	0.04	0.03	0.06	0.04
Human serum	0.04	0.02	0.05	0.04
HCV core peptide	0.03	0.04	0.06	0.04
PBS	0.02	0.02	0.04	0.04

Positive control: Polyclonal immunized mouse serum.
Negative control: non immunized mouse serum.
PBS: phosphate-buffered saline.
OD: Optical density at 450 nm.

(1G10) and 10B2 (clone 3A6) hybridomas was carried out "*in vivo*" using BALB/c mice and the ascitic fluid gave also good reactivity with HCV E1 antigen.

Specific Antigen Identified

ELISA showed that the antibodies from hybridoma cell lines 10C7 (clone 1G10) and 10B2 (clone 3A6) reacted with HCV E1 synthetic peptide free only. Antibodies 10C7 (clone 1G10) and 10B2 (clone 3A6) reacted better with HCV E1 synthetic peptide conjugated with or without BSA using ELISA. Dot-ELISA was also used to rapidly screen the monoclonal antibodies (10C7 clone 1G10), 10B2 clone 3A6) against our immunized peptide. Monoclonal antibodies 10C7 and 10B2 gave strong reactivity with the HCV E1 synthetic peptide (immunized peptide). Positive mouse serum gave positive staining with Dot-ELISA, while negative mouse serum gave no reaction with HCV E1 synthetic peptide, Figure 3.

Subclass Analysis of anti-E1 mAbs

Hybridoma cell culture supernatants were used as the primary antibody to identify the subclass of mAbs. We found that (10C7 clone (1G10) hybrid cells produce monoclonal antibody with heavy chains: IgG2b isotype light chains and kappa isotype while 10B2 (clone 3A6) hybrid cells produce monoclonal antibody with heavy chains: IgG1 isotype light chains and kappa isotype, Figure 4.

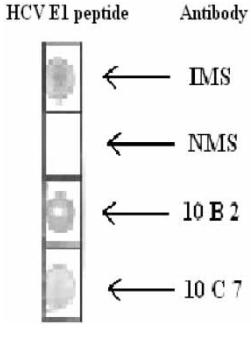

Figure 3. Dot-ELISA screening of monoclonal antibodies 10C7 (clone 1G10) and 10B2 (clone 3A6) against HCV E1 peptide. NMS: normal mouse serum. IMS: Immunized mouse serum.

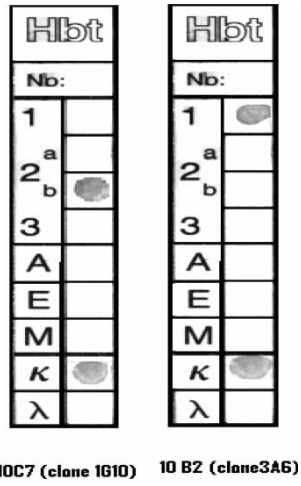

Figure 4. Subclass analysis of the monoclonal antibodies. Mouse monoclonal antibodies (10C7 clone (1G10) and 10 B2 (clone 3A6) were immunoglobulin G2b, kappa chain [IgG2b, and (_immunoglobulin G1, kappa chain [IgG1)], respectively.

Identification of HCV-E1 Antigen in Sera of HCV Patients by Western Blotting

Sera of HCV infected patients who have been confirmed to be infected with the HCV using RT-PCR and had high titer of E1 viral antigen were used for Western Blot analysis. Sera of healthy individuals were included as negative controls. Results showed that treatment of resolved antigens from infected patients with mouse monoclonal antibody raised against HCV E1 peptide resulted in visualization of an immunogenic protein band around 38-kDa that was undetectable in sera from the healthy controls (Figure 5). Another band at ~21-kDa was visualized in positive sera which may be a degradation part from the original band.

Detection of Serum HCV-E1 Antigen by ELISA

The well established ELISA technique was adapted to detect the target marker HCV-E1 antigen in sera of infected individuals (Table 2). We have found 90% agreement between the results of qualitative detection of HCV-RNA using PCR tests and the HCV-E1 antigen detection using ELISA for a group of 100 HCV infected individuals and 84% agreement between the results for a group of 25 HCV free healthy volunteers.

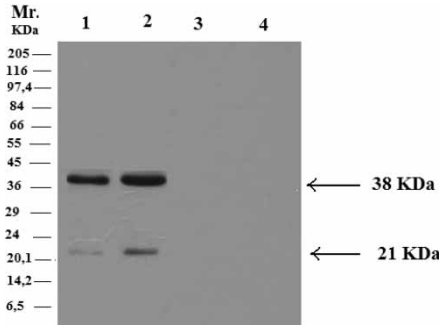

Figure 5. Western blot analysis of serum samples from *Hepatitis C virus* infected and non-infected individuals with monoclonal antibody against HCV E1. The HCV E1 mAbs identified a circulating antigen at ~38 kDa in serum samples of infected patients in addition to a degradation product of ~21 kDa. Lanes 1 and 2: serum samples from two individuals infected with HCV. Lanes 3 and 4: serum samples from two non-infected healthy individuals. Molecular mass bands are not shown but are indicated by arrows.

DISCUSSION

HCV remains a large health care burden to the world. Incidence rates across the world fluctuate and are difficult to calculate, given the asymptomatic, often latent, nature of the disease prior to clinical presentation. Prevalence rates across the world have changed as well with more countries aware of transfusion-related hepatitis C and more and more evidence supporting intravenous drug use as the leading risk factor of spread of the virus.[12] In the present

Table 2. Detection of HCV E1 antigen using ELISA in comparison with qualitative HCV RNA detection using PCR test in sera of 100 Egyptian individuals

HCV–RNA detection using RT-PCR[a]	HCV E1 antigen using ELISA[b]			
	No positive	Positive	Negative	%
Positive HCV RNA	100	90 (TP)	10 (FN)	90
Negative HCV RNA	25	4 (FP)	21 (TN)	16

Abbreviation: TP, true positive; FP, false positive; TN, true negative, FN, false negative

[a]The gold standard for the diagnosis of HCV infection.

[b]Sensitivity (%) = TP/(TP + FN) = 90/(90 + 10) × 100 = 90%.

Specificity (%): TN/TN + FP) = 21/(21 + 4) × 100 = 84%.

PPV (%) = TP/(TP + FP) = 90/(90 + 4) × 100 = 95.7%.

NPV (%) = TN/(TN + FN) = 21/(21 + 10) × 100 = 67.7%.

Efficiency of ELISA (%) = (TP + TN)/total = 90 + 21/125 = 88.8%.

study, we established two hybrid cell lines secreting monoclonal antibodies (mAbs) against HCV E1 synthetic peptide and have characterized their properties. We used amino acid sequence GHRMAWDMM stretch within the N-terminal region of the E1 protein derived from several reported HCV isolates, as demonstrated in our recent study.[6] We previously used this synthetic peptide for immunization of New Zealand rabbits; the reactivity of hyper-immune E1 antibody was confirmed by ELISA and Western blot for detection of HCV E1 antigen in infected blood lymphocytes,[7] infected HepG2 cells,[8] and infected sera.[9] Polyclonal antibody against this peptide showed blocks HCV infection to target cells, suggesting the involvement of this epitope in virus binding and entry.[6] Also, we suggested that isolation of similar humanized antibodies that block virus binding and entry will be useful in providing potential therapeutic reagents and for vaccine development. In this study, we used the same synthetic peptide sequence (GHRMAWDMM) of HCV E1 for production of mouse monoclonal antibodies by hybridoma technology. Several studies used mouse monoclonal antibodies to neutralize the cell culture infectious HCV.[13] Dreux et al.[14] showed that monoclonal antibodies targeted to HCV-E1 efficiently neutralize infectious pseudo-particles (HCVpp) and cell culture-grown genuine HCV (HCVcc) in the presence of human serum. Recently, Eren et al.[15] used human monoclonal antibodies (HuMAbs) (HCV-AB 68 and HCV-AB 65) against the E2 envelope protein of HCV which were developed and tested for the ability to neutralize the virus and prevent human liver infection. The demonstrated neutralizing activities of HCV-AB 68 and HCV-AB 65 suggest that they have the potential to prevent reinfection in liver transplant patients and to serve as prophylactic treatment in post exposure events. Also, several works used monoclonal antibodies for diagnosis or monitoring antiviral activity of HCV.[16–18] In the present work, we had established two hybridoma cell lines secreting mAbs (10C7 clone (1G10), and 10 B2 (clone 3A6)) against HCV E1 synthetic peptide. We found that mouse monoclonal antibodies (10C7 clone (1G10) and 10 B2 (clone 3A6)) were immunoglobulin G2b, kappa chain [IgG2b], and immunoglobulin G1, kappa chain [IgG1)], respectively. Indirect ELISA demonstrated that both mAbs had no cross-reactivity with bovine serum albumin or other HCV proteins, confirming its specificity and reactivity to HCV E1 antigen. In the present study, we have identified a target HCV E1 antigen in sera of infected individuals using mouse monoclonal antibodies produced by the hybrid cell line developed against synthetic peptide derived from HCV E1 region. The target HCV E1 antigen was detected in sera using enzyme immunoassay. The well established ELISA based on the in-house produced mouse monoclonal antibody by hybridoma technique showed 90% agreement with HCV RNA detection using RT-PCR among HCV infected individuals and 84% agreement among non-infected individuals. Detection of HCV antigens were demonstrated by several investigators[9,16,19–21] and this is in agreement with our results. The detected false positive results may be attributed to the low level of HCV

RNA viremia in these sera, which may be lower than the detection limit of the RT-PCR or may be due to other unknown factors. The false negative results of the developed ELISA test may be explained by assuming that the HCV E1 target antigen level among false negative samples may be too low to be detected. Also, there may be a presence of immune-complex and it may be necessary to dissociate the immune complexes to achieve a higher sensitivity in the immunoassay.[22,23] However, the serum samples will be pretreated with detergents to increase the developed assay sensitivity. This pretreatment inactivates the antibodies and, simultaneously, the antigens are released and the epitopes are exposed. All the HCV RNA positive sera are predominantly genotype 4. However, the evaluation of the assay using sera from patients infected with various HCV genotyping will be performed. Generally, clearance of HCV RNA after treatment with interferon alpha is associated with disappearance of core protein from serum.[24] Data of Tanaka et al.[25] suggested that antigenemia and viremia of HCV appear and disappear in parallel. Further studies regarding the detection of the HCV E1 target antigen, before and after treatment, will be investigated. Future research in this field will need to be continued. We will need to continue to evaluate the use of these antibodies for detection of and study of the HCV life cycle and to study its efficacy as immune prevention agents. Better prevention, screening, and treatment methods for Hepatitis C virus all need to be elucidated. In conclusion, two hybridoma cell lines secreting mAbs against HCV E1 synthetic peptide with a sequence of GHRMAWDMM have been established successfully, which may provide a useful tool for the antigenic analysis, serological diagnosis, and the evaluation of HCV vaccine.

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