Journal of Medical Virology

Design and Production of a Multiepitope Construct Derived From *Hepatitis E Virus* Capsid Protein

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The aim of this study was to design a high density multiepitope protein, which can be a promising multiepitope vaccine candidate against *Hepatitis E virus* (HEV). Initially, conserved and antigenic helper T-lymphocyte (HTL) epitopes in the HEV capsid protein were predicted by in silico analysis. Subsequently, a multiepitope comprising four HTL epitopes with high-affinity binding to the HLA molecules was designed, and repeated four times as high density multiepitope construct. This construct was synthesized and cloned into pET-30a (+) vector. Then, it was transformed and expressed in *Escherichia coli* BL21 cells. The high density multiepitope protein was purified by Ni-NTA agarose and concentrated using Amicon filters. Finally, the immunological properties of this high density multiepitope protein were evaluated in vitro. The results showed that the high density multiepitope construct was successfully expressed and purified. SDS-PAGE and Western blot analyses showed the presence of a high density multiepitope protein band of approximately 33 kDa. Approximately 1 mg of the purified protein was obtained from each liter of the culture media. Moreover, the purified multiepitope protein was capable of induction of proliferation responses, IFN-γ ELISPOT responses and IFN-γ and IL-12 cytokines production in a significant level in peripheral blood mononuclear cells (PBMCs) isolated from HEV-recovered individuals compared to the control group. In conclusion, the newly produced multiepitope protein can induce significant T helper type 1 responses in vitro, and can be considered as a novel strategy for the development of HEV vaccines in the future. ***J. Med. Virol.***

KEY WORDS: *hepatitis E virus*; ORF2; cellular immune responses; cell proliferation assay; IFN-γ ELISPOT; cytokines

INTRODUCTION

Hepatitis E virus (HEV) is a positive-stranded RNA virus which has been classified as the member of the genus *Hepevirus* in the family *Hepeviridae* [Yamashita et al., 2009]. At least four major HEV genotypes have been identified, which have different geographical distributions and host ranges [Xing et al., 2011]. Despite classification into four geographically distinct genotypes, the HEV strains antigenically belong to a single serotype and share cross-reacting epitopes due to the high degree of sequence conservation [Wang and Zhuang, 2004; Xing et al., 2011]. Genotype 1 includes most of human HEV strains which is endemic in Asia and North Africa, genotype 2 is identified in human and includes the Mexican strain and few strains of African countries. Genotypes 3 and 4 have been identified in pigs and other animals; however, humans can be infected with these two

The authors declare there are no conflicts of interest in the content of this study.

Grant sponsor: Infectious and Tropical Diseases Research Center of Ahvaz Jundishapur University of Medical Sciences; Grant number: 91111.

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Accepted 26 January 2015

DOI 10.1002/jmv.24171
Published online in Wiley Online Library (wileyonlinelibrary.com).

genotypes too. Genotype 3 predominates in industrialized countries, and genotype 4 is endemic in Asia, particularly China, Taiwan, and Japan [Emerson and Purcell, 2001; Yamashita et al., 2009].

HEV causes acute hepatitis in one-third of the world's population [Zhu et al., 2010], with mortality rate of 1–15% in the general population [Xing et al., 2011]. However, sporadic and epidemic outbreaks of the acute hepatitis usually lead to a self-limited disease, but the infection is potentially fatal and causes fulminant hepatitis in susceptible individuals such as pregnant women and patients with underlying liver disease [Zhu et al., 2010; Xing et al., 2011], and has high mortality rate of up to 30% among pregnant women [Xing et al., 2011]. Therefore HEV is a significant public health problem worldwide especially in endemic areas and primary prevention of the HEV infection seems essential.

Currently, no protective vaccine against HEV infection has been licensed and primary prevention remains limited [Wang and Zhuang, 2004]. Development of an effective vaccine, which can significantly reduce the prevalence of this disease, is a desirable goal. Killed and live attenuated vaccines are not available due to the lack of a permissive cell culture system for HEV replication [Emerson and Purcell, 2001]. Therefore the main focus for vaccine design is on molecular approaches, especially expression of viral proteins in different expression systems [Emerson and Purcell, 2001; Jimenez de Oya et al., 2012].

The HEV genome (~7.2 kb) contains three partially overlapping open reading frames (ORFs), including ORF1, ORF2 and ORF3 [Zhou et al., 2004; Jimenez de Oya et al., 2012]. ORF2, which encodes capsid protein, is highly conserved and immunogenic among HEV species [Emerson and Purcell, 2001]. Due to hydrophobicity and insolubility of full-length capsid protein (72 kDa) [Tsarev et al., 1994; Zhang et al., 2001], new generation vaccines such as multiepitope vaccines are of particular interest. Therefore, the present study was undertaken to design a high density multiepitope protein compromising four HTL epitopes with high-affinity binding to the HLA molecules using the in silico analysis, and to evaluate the immunological properties of this protein in vitro. Overall, this study represents a novel strategy for the development of HEV vaccines in the future.

MATERIALS AND METHODS

Rational Design of a High Density Multiepitope Construct From HEV ORF2 Protein

The amino acid sequences of the ORF2 from Standard strains of HEV genotype 1 including Pakistan/Sar-55 strain (accession number P33426), China strain (accession number Q81871), Burma strain (accession number P29326), Myanmar strain (accession number Q04611) and India strain (accession number Q68985) were obtained from Uniprot Knowledgebase (www.uniprot.org). The sequences were aligned by MEGA software version 4.0 (Biodesign Institute, Tempe, AZ) [Tamura et al., 2007], and then the conserved sequences among these Strandard strains were determined. The highly conserved HEV-derived HLA-DR-restricted HTL epitopes of these strains were predicted by SYFPEITHI online software (http://www.syfpeithi.de/home.htm). HLA-DR binding affinity of the predicted epitopes was determined by IEDB Analysis Resource online program (http://tools.immuneepitope.org/analyze/html/mhc_II_binding.html). Genetic diversity of MHC molecules is very high in human population; therefore HTL epitopes with high population coverage are needed for this study. Four epitopes with high-affinity to the most common HLA-DR alleles in Iran and world population were selected. Therefore it is predicted that this construct could cover the most world population. The four HTL epitopes (15 amino acids) derived from HEV capsid protein including HLA-DRB1*1501 (A150–164), HLA-DRB1*0701 (B320–334), HLA-DRB1*1101 (C370–384), HLA-DRB1*0401 and HLA-DRB3*0101 (DR52) (D491–505)-restricted epitopes were binded to each other as a linear multiepitope (ABCD). To avoid creation of junctional epitopes, the construct was designed with amino acid spacer sequences (Gly–Pro–Gly–Pro–Gly) between sequential HTL epitopes [Livingston et al., 2002; Depla et al., 2008]. The four times repeated multiepitope was constructed as high density multiepitope (Fig. 1A). Based on *E. coli* codon usage, the nucleotide sequence of the high density multiepitope gene was optimized using GenScript Rare Codon Analysis Tool (GenScript, Piscataway, NJ) for efficient expression. In order to simplify the purification of the target protein, a sequence encoding 8-His tag was added at the C-terminal of the optimized gene followed by adding two stop codons (TAA and TAG) to terminate protein synthesis. Two restriction-enzyme digestion sites, NdeI and XhoI, were placed in the both ends of the codon-optimized gene to subclone it into the pET30a (+) vector.

NdeI digestion site (CATATG) with starting codon (ATG) was inserted at the N-terminal of the construct. The XhoI digestion site (CTCGAG) was inserted at the C-terminal of the construct after the second termination codon. In silico digestion was performed using Clone Manager Basic software version 9 (Sci-Ed Software, Cary, NC), and was checked by Vector NTI software (Invitrogen, Carlsbad, CA). Finally, the optimized high density multiepitope sequence was synthesized and cloned into the commercial cloning vector, pBluescript II SK (+) by Biomatik Company (Biomatik Corporation, Cambridge, Canada).

Subcloning and Construction of the Recombinant Plasmid

Both pBluescript II SK (+) vector carrying the high density multiepitope gene (pBluescript II SK-high

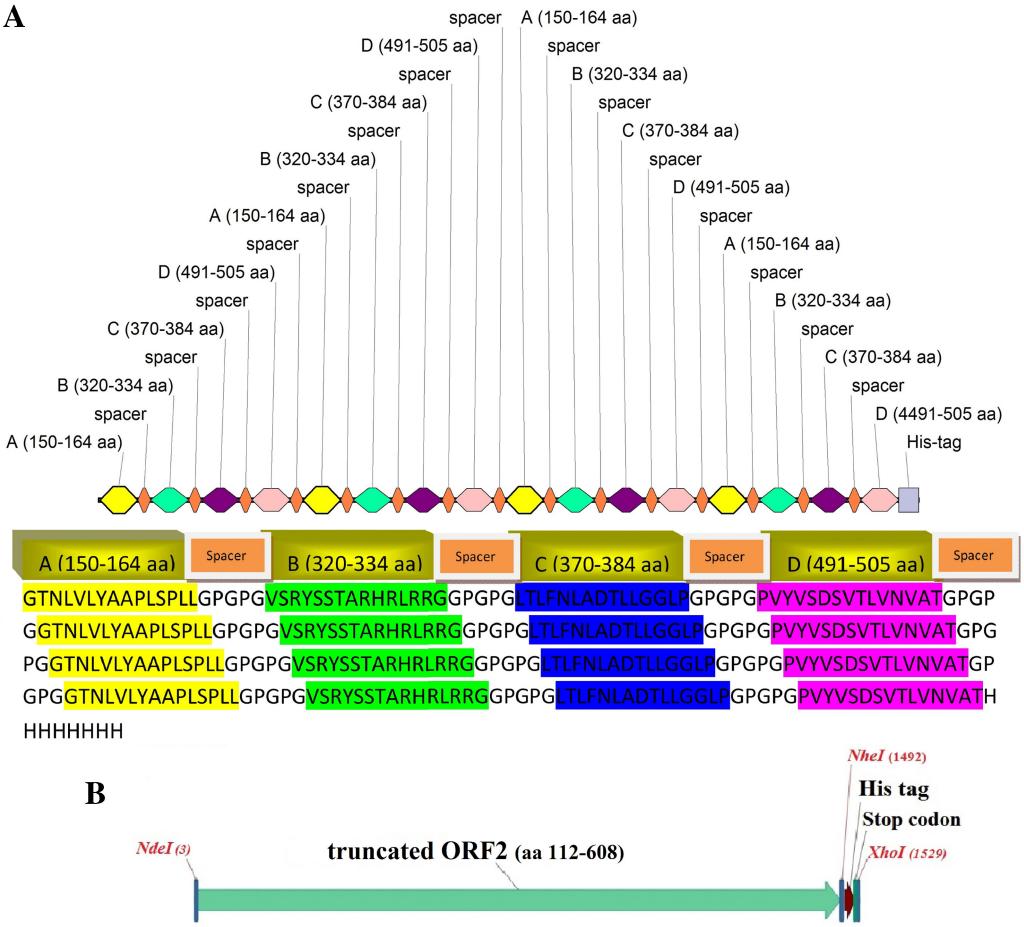

Fig. 1. **A**: Schematic presentation of high density multiepitope construct. The amino acid sequence of four HTL epitopes from HEV capsid protein and GPGPG amino acid spacers between them in the high density multiepitope construct are presented. **B**: Schematic presentation of truncated ORF2 (aa 112–608).

density multiepitope) and the pET-30a (+) plasmid (Novagen, Madison, WI) were digested by NdeI and XhoI restriction enzymes (New England BioLab, Ipswich, MA). After ligation by T4 DNA ligase (New England BioLab, Ipswich, MA), the recombinant plasmid pET30a-high density multiepitope was generated and transformed into *E. coli* DH5α competent cells (Novagen) by electroporation [Chassy et al., 1988]. The subcloning was confirmed by colony PCR, restriction-enzyme digestion, and DNA sequencing. The recombinant plasmid was then transformed into competent *E. coli* BL21 (DE3) cells to enhance protein expression (Novagen).

Protein Expression and Purification

E. coli BL21 (DE3) cells containing the recombinant plasmid were cultured overnight, then inoculated in Terrific Broth (HiMedia, Mumbai, India) supplemented with kanamycin (50 mg/L) in a 1:100 volumetric ratio, and grown with shaking at 250 rpm at 37°C until the optical density at 600 nm (OD 600) reached 0.5. The bacterial culture was induced by adding various concentrations of IPTG (0.1–1 mM) (Sigma–Aldrich, St. Louis, MO), and grown with shaking for different induction times (2, 4, 6, 8, and 16 hr) at different induction temperatures (37°C, 30°C, and 25°C) to determine the optimal conditions of protein expression. After the large-scale protein expression, the target protein was purified by Ni-NTA (Qiagen, Hilden, Germany) under denaturing conditions using 8 M urea [Taherkhani et al., 2014]. The purified protein was dialyzed in PBS containing 10% glycerol with a linear urea gradient of 6, 4, and 2 M at 4°C for 8 hr in order to decrease the amount of urea, and concentrated using Amicon Ultra-4 Centrifugal Filter Unit (EMD Millipore, Billerica, MA). SDS-PAGE and Western blot were performed to confirm the presence of the target protein [Laemmli, 1970; Hao et al., 2013; Taherkhani et al., 2014]. Concentration of the protein was measured by the Bradford method [Bradford, 1976]. To remove bacterial

endotoxin contamination in the purified protein, Toxin Eraser Endotoxin Removal kit was used according to the manufacturer procedure (GenScript).

Truncated ORF2 Protein

The truncated ORF2 protein (aa 112–608) of HEV strain sar-55 (Fig. 1B) was expressed in *E. coli* BL21 host cells using the recombinant plasmid pET-30a-ORF2 (aa 112–608) and purified by Ni-NTA (Qiagen) as described previously [Taherkhani et al., 2014].

Study Patients

This protocol was approved by the ethical committee of Ahvaz Jundishapur University of Medical Science. A statement on Ethical Committee approval and the informed consent was taken from all participants. Ten recovered individuals from acute hepatitis E infection (6 males and 4 females; mean age ± SD, 40.30 ± 13.76 years) with positive anti-HEV IgG antibody were enrolled in this study. The control group consisted of 12 healthy individuals (7 males and 5 females; mean age ± SD, 38.08 ± 15.35 years) with no history of acute hepatitis, and with negative anti-HEV IgM/IgG antibodies. The commercial ELISA kits (HEV IgG and HEV IgM ELISA kits, DIA.PRO, Milan, Italy) were used for all the participants. All the participants were negative for hepatitis B surface antigen, anti-HAV IgM antibody and anti-HCV antibodies (HBs Ag ELISA kit, HAV IgM ELISA kit, HCV Ab ELISA kit, DIA.PRO) with normal ALT level.

Separation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) of the each individual were isolated from 10 ml heparinized venous blood by density gradient centrifugation using Ficoll-Hypaque (Lymphoflot, Biotest, Dreieich, Germany). The isolated PBMCs were washed and resuspended in complete RPMI 1640 medium (Invitrogen). The viability of the cells was assessed by Trypan blue staining.

Cell Proliferation Assay Using Non-Radioactive MTT Assay

The cell proliferation assay was performed using non-radioactive MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay [Majumdar et al., 2013]. In brief, approximately 1×10^5 cells/well of PBMCs of each sample in RPMI 1640 and 10% FCS were added to four wells of round-bottom 96-well plates in total volume of 180 μl/well, stimulated with 20 μl/well of truncated ORF2 protein (10 μg/ml), high density multiepitope (10 μg/ml) and Phytohemagglutinin (PHA) (5 μg/ml) (Sigma–Aldrich) separately, and incubated at 37°C for 4 days. The unstimulated well of each sample was used as negative control. RPMI 1640 (20 μl) was used as blank. After 4 days, 10 μl of MTT solution (5 mg/ml) was added to each well and incubated at 37°C for 4 hr. Then, 100 μl of isopropanol containing 0.04 N HCl was added to each well and mixed thoroughly. Finally, the absorbance of the wells was read at 570 nm with a reference wavelength of 630 nm by an ELISA reader (Tecan Sunrise ELISA Reader; Tecan Trading AG, Mannedorf, Switzerland). The results were showed as proliferation index (PI), calculated as OD570 stimulated sample/OD570 unstimulated sample.

Enzyme-Linked Immunosorbent Spot (ELISPOT) Assay

The ELISPOT assay for HEV-specific IFN-γ-secreting cells was carried out by Human IFN-γ ELISPOT Ready-SET-Go kit (eBioscience, San Diego, CA) according to manufacturer's protocol. Briefly, a 96-well PVDF bottomed ELISPOT plate (Millipore, Bedford, MA) was coated with 100 μl/well of Capture Antibody solution and incubated overnight at 4°C. Approximately 2×10^5 cells/well of PBMCs of each sample suspended in RPMI 1640 and 10% FCS along with 10 μg/ml high density multiepitope protein and 10 μg/ml truncated ORF-2 protein separately were added to 2 wells in total volume of 150 μl/well. For negative control unstimulated PBMCs was used. For positive control, 5 μg/ml of PHA (Sigma–Aldrich) was used. After 24-hr incubation at 37°C, cells and medium were decanted from the plate and the plate was washed three times with ELISPOT Wash Buffer. 100 μl of Biotinylated Detection Antibody was added to each well and incubated overnight at 4°C. After incubation, the plate was emptied and washed as described above. 100 μl/well of Avidin–Horseradish Peroxidase reagent was added, and the plates were incubated at room temperature for 45 min. After incubation, the plates were washed three times with ELISPOT wash buffer and two times with 1X PBS; then, 100 μl/well of freshly prepared AEC (3-amino-9-ethyl carbazole) substrate solution (Sigma–Aldrich) was added to the wells and incubated at room temperature until dark purple spots appears. The reaction was stopped by washing wells three times with 200 μl/well distilled water. Plate was air-dried and the number of IFN-γ–producing cells in each well was counted by a dissection stereoscope (Nikon, Tokyo, Japan) and expressed as spot-forming cells (SFCs) per 10^5 cells.

Cytokine Assay

At a concentration of 1×10^5 cells/well, PBMCs of each sample in RPMI 1640 and 10% FCS were added to the four wells of 96-well plates, and stimulated with truncated ORF2 protein (10 μg/ml), high density multiepitope protein (10 μg/ml), and PHA (5 μg/ml) separately at 37°C. The unstimulated PBMCs of each sample were used to evaluate spontaneous production of cytokines. Culture supernatants were collected after 48 hr (24 hr for IL-12). Levels of four cytokines

(IL-12 p70, IFN-γ, IL-4 and IL-10) were measured in duplicate by commercial ELISA kits (eBioscience) according to the manufacturer's instructions. The ELISA results were expressed as picograms per milliliter (pg/ml).

Statistical Analysis

Statistical analysis was performed using SPSS 17 software (SPSS, Chicago, IL) and P values of less than 0.05 were considered statistically significant. Discrete variables were compared using the Pearson's chi-square test or fisher's exact test. Intergroup comparisons (recovereds vs. controls) were performed using Independent T Test or Mann–Whitney U test. Intragroup comparisons (High density multiepitope versus ORF2) were made by Paired T Test or Wilcoxon test. All data were presented as mean ± SE.

RESULTS

Preparation of High Density Multiepitope Protein

The high expression of the protein was induced by IPTG at a final concentration of 1 mM at 30°C for 4 hr. SDS-PAGE and Western blotting analysis of the resultant protein showed a protein band of approximately 33 kDa corresponding to high density multiepitope (Fig. 2). Approximately 1 mg of purified protein was obtained from each liter of culture media.

Cell Proliferative Responses to High Density Multiepitope Protein and Truncated ORF2 Protein

The cell proliferative responses in HEV-recovered group following stimulation with high density multiepitope protein and truncated ORF2 protein were found higher than control group ($P < 0.001$); and these responses were observed higher with high density multiepitope protein than truncated ORF2 protein in HEV-recovered group ($P = 0.013$) (Table I, Fig. 3).

IFN-γ ELISPOT Responses to High Desity Multiepitope Protein and Truncated ORF2 Protein

IFN-γ ELISPOT responses to high density multiepitope protein and truncated ORF2 protein were found significantly higher in HEV-recovered individuals than control group ($P < 0.001$). These responses to high density multiepitope protein were observed slightly higher than truncated ORF2 protein in HEV-recovered group, with no significant observation ($P = 0.220$). Table I and Figure 4 show the frequency of IFN-γ-secreting cells following stimulation with high density multiepitope protein and truncated

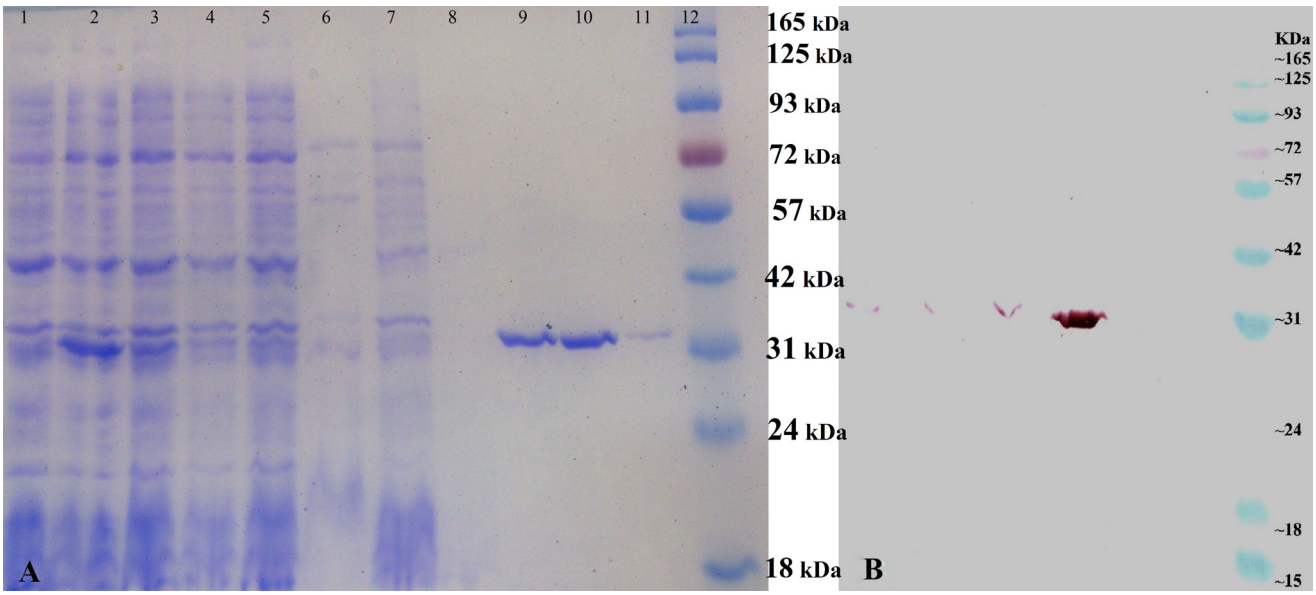

Fig. 2. **A**: SDS-PAGE analysis of the expressed and purified high density multiepitope protein in *E. coli*. The high density multiepitope protein band is visualized at approximately 33 kDa. Lane 1, uninduced control; Lane 2, induction with 1 mM IPTG for 4 hr; Lane 3, induction with 1 mM IPTG for 8 hr; Lane 4,5, flow-through; Lane 6–8, washing steps of the Ni column; Lane 9–11, the first, the second, and the third purified eluates of high density multiepitope protein from the Ni column; Lane 12, prestained protein ladder (Cinagen, Tehran, Iran). **B**: Western blot analysis of the expressed and purified high density multiepitope protein in *E. coli*. The high density multiepitope protein band is visualized at approximately 33 kDa. Lane 1, prestained protein ladder (Cinagen); Lane 2, flow-through; Lane 3, wash; Lane 4–7, the first, the second, the third, and the fourth eluates.

TABLE I. Levels of Cytokines, IFN-γ ELISPOT, and Cell Proliferative Responses to High Density Multiepitope Protein and Truncated ORF2 Protein in HEV-Recovered Individuals and Control Group.

	IFN-γ	IL-12	IL-10	IL-4	ELISPOT	Cell proliferation
Control group (n = 12)						
Unstimulated	7.70 ± 1.26	9.12 ± 0.61	11.90 ± 2.24	3.00 ± 0.408	1.58 ± 0.57	
ORF2	14.28 ± 1.84	17.07 ± 2.63	19.16 ± 2.77	4.63 ± 0.54	3.00 ± 1.51	1.40 ± 0.032
Multiepitope	15.63 ± 1.67	18.08 ± 2.14	17.08 ± 2.92	4.79 ± 0.41	2.00 ± 0.55	1.30 ± 0.038
PHA	408.08 ± 14.76	105.58 ± 8.50	205.50 ± 30.45	11.56 ± 1.36	215 ± 29.76	2.80 ± 0.087
Recovered group (n = 10)						
Unstimulated	11.82 ± 2.01	12.50 ± 2.17	16.30 ± 3.19	3.20 ± 0.37	2.20 ± 0.77	
ORF2	136.86 ± 17.46	106.70 ± 13.41	24.80 ± 6.58	5.31 ± 0.54	58.90 ± 18.31	1.90 ± 0.057
Multiepitope	142.70 ± 16.21	115.40 ± 16.71	25.30 ± 5.23	4.11 ± 0.38	63.00 ± 16.96	2.20 ± 0.063
PHA	415.70 ± 22.59	100.60 ± 8.91	197.40 ± 32.45	10.90 ± 1.29	226.80 ± 41.0	2.70 ± 0.063
P-value*	0.114 (NS)	0.742 (NS)	0.177 (NS)	0.824 (NS)	0.811 (NS)	0.074 (NS)
P-value**	0.132 (NS)	0.335 (NS)	0.839 (NS)	0.127 (NS)	0.220 (NS)	0.013
P-value***	<0.001	<0.001	0.446 (NS)	0.395 (NS)	<0.001	<0.001
P-value****	<0.001	<0.001	0.168 (NS)	0.251 (NS)	<0.001	<0.001

The levels of cytokines are presented as picograms per milliliter (pg/ml), the results of ELISPOT assay are expressed as spot forming cells per 10^5 cells (SFC/10^5 cells), the results of cell proliferation assay are shown as proliferation index (PI).
All Data are shown as mean ± SE.
NS = non-significant.
*ORF2 vs. high density multiepitope in control group.
**ORF2 vs. high density multiepitope in recovered group.
***Recovered vs. control regarding ORF2.
****Recovered vs. control regarding high density multiepitope.

ORF2 protein in HEV-recovered individuals and control group.

HEV-Specific Cytokine Responses

The levels of IFN-γ and IL-12 p70 responses to truncated ORF2 protein and high density multiepitope in the recovered group were found significantly higher compared to control group ($P < 0.001$). However, the levels of these stimulated cytokines with high density multiepitope protein were found higher than truncated ORF2 protein with no significant observation. The levels of specific IL-10 for high density multiepitope protein and truncared ORF2 protein were found moderate among the HEV-recovered group, while the levels of specific IL-4 for the both proteins were low (Table I, Fig. 5).

DISCUSSION

A better understanding of the HEV-specific cellular immune responses may help us in designing and development of vaccine for the prevention and control of the HEV infection. Although some progress has

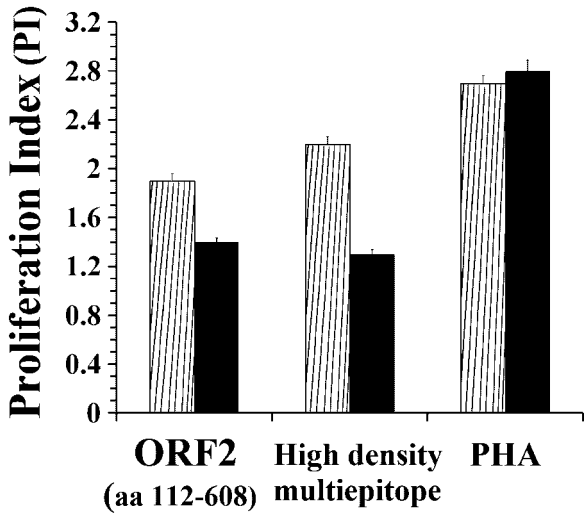

Fig. 3. Cell proliferative responses to high density multiepitope protein and truncated ORF2 protein in HEV-recovered individuals and control group. The results are shown as mean ± SE PI (proliferation index). Black (■) and hatched (▨) bars represent control group and HEV-recovered group, respectively.

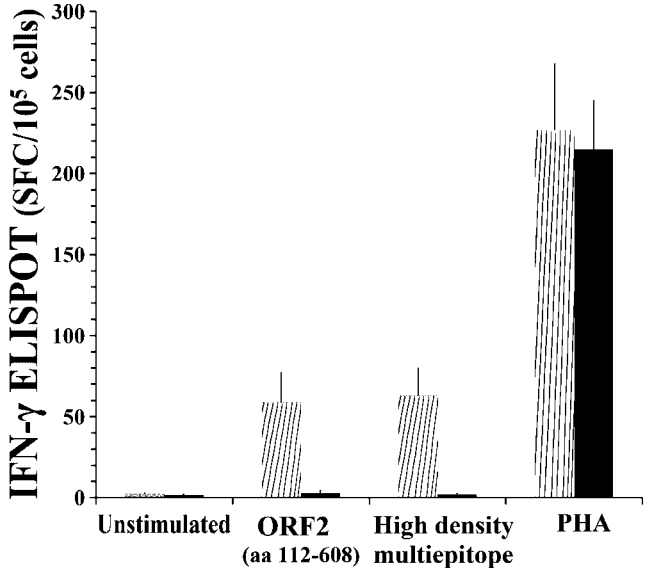

Fig. 4. IFN-γ ELISPOT responses to high desity multiepitope protein and truncated ORF2 protein in HEV-recovered and control groups. Data are presented as spot forming cells per 10^5 cells (SFC/10^5 cells). Black (■) and hatched (▨) bars represent control group and HEV-recovered group, respectively.

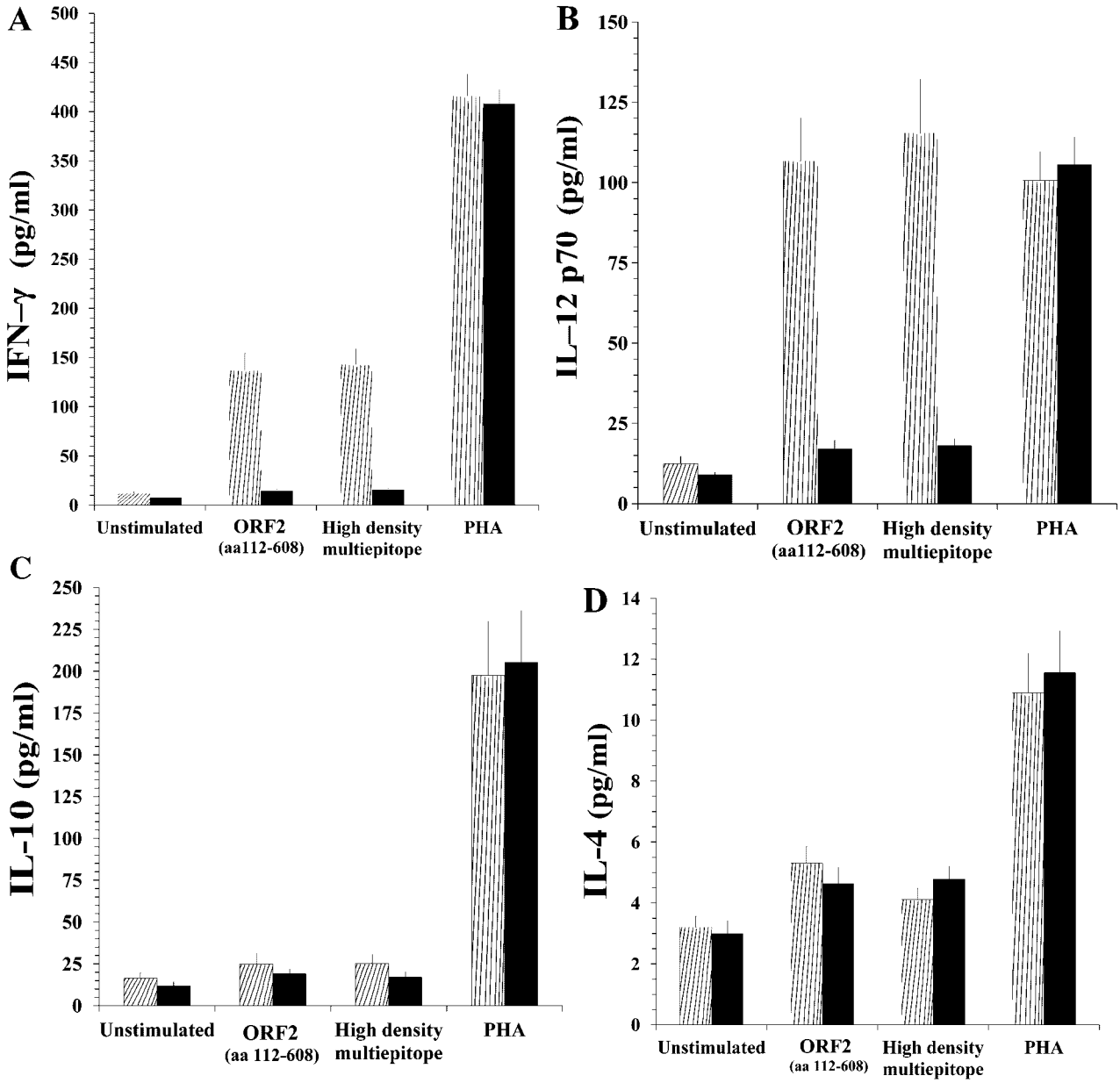

Fig. 5. The levels of IFN–γ (**A**), IL–12 p70 (**B**), IL–10 (**C**), and IL–4 (**D**) responses to truncated ORF2 protein and high density multiepitope protein in HEV-recovered and control groups. Data are presented as picograms per milliliter (pg/ml). Black (■) and hatched (▨) bars represent control group and HEV-recovered group, respectively.

been achieved in developing HEV vaccine, but due to some limitations in current approach for vaccine preparation such as the duration of the response and cost effectiveness of vaccine, the attempt was made to develop an alternative HEV vaccine [Jimenez de Oya et al., 2012].

The virus-specific T-helper cell immune responses have been shown to be essential for controlling and clearing viral infection by activating cytotoxic T cells and producing cytokines especially INF-γ which can suppress viral replication. Thus, viral proteins comprising helper T-lymphocyte (HTL) epitopes may be useful as protective vaccines [Aggarwal et al., 2007]. It has been shown that HEV capsid protein has several immunogenic epitopes [Gu et al., 2004]. Shata et al. identified dominant and subdominant epitopes of HEV capsid protein such as amino acids 271–286, 199–214, 463–478, 583–598, and 631–646 [Shata et al., 2007]. Aggarwal et al. mapped CD4 T-cell epitopes in HEV ORF2 protein, and found the immunodominant T-cell epitopes of capside protein are encompassed amino acids 73–156, 289–444, and 505–588 [Aggarwal et al., 2007]. Khudyakov et al. reported the antigenic regions in HEV ORF2 protein

are encompassed amino acids 319–340, 631–648, and 641–660 [Khudyakov et al., 1993]. Selection of the epitopes is the most important part of the design of the epitope-based vaccines that can guarantee the effectiveness and extent of the desired immune responses.

This study focused on the consereved epitopes with high-affinity binding to the different HLA molecules to cover the majority of the human population, on this basis the multiepitope protein comprising these epitopes was constructed. This multiepitope design is a new strategy and may counter the limitations present in current vaccine against HEV, but it requires further investigation. To date, no application of multiple HLA-DR-restricted epitopes was reported to induse HTL responses against HEV infection. Oany et al. designed a multiepitope vaccine candidate compromising B cell and T cell epitopes from spike protein of human coronavirus by in silico analysis. In their study, sequences of the spike proteins were collected from the UniProtKB database and the consereved epitopes with high-affinity binding to the different HLA molecules were predicted using in silico tools to cover the majority of the world population. This computational design was a novel study to determine antigenic epitopes in spike protein and to design an epitope-based vaccine against human coronavirus [Oany et al., 2014].

These type of vaccines were reported to have many advantages including the stability, lack of carcinogenic, large scale production with the least equipments, no need for cold chain storage, the ability to stimulate the immune system against a wide variety of strains and genotypes of a microorganism, and use in different races or high population coverage [Elliott et al., 1999; Livingston et al., 2002; Sette and Fikes, 2003; Jackson et al., 2006]. Meanwhile, some disadvantages like the creation of junctional epitopes in the sequential arrangement of the epitopes have limited using these constructs [Livingston et al., 2002; Depla et al., 2008]. Junctional epitopes is created by the juxtaposition of two epitopes, and can suppress the immune responses to the main epitopes [Livingston et al., 2002]. This problem was overcome through application of the spacer residues between the epitopes to improve appropriate epitope processing. Furthermore, it has been reported that if the epitope is expressed with high density will enhance protective immunity [Liu et al., 2004; Lu et al., 2008]. Lu et al. designed constructs consisting of various V3 epitope densities from HIV-1 gp120, and found that high epitope density in one single protein molecule could enhance protective immunity [Lu et al., 2008]. Liu et al. reported that high epitope density from M2 protein of influenza virus enhanced specific humoral immunity against flu virus [Liu et al., 2004].

Vaccine development is dependent not only on selecting antigens and epitopes but also on defining the immune responses against target epitopes. By evaluating the antigenicity and immunogenicity of the high density multiepitope protein, it was indicated that the high density multiepitope protein stimulates slightly stronger T helper cell responses compared to truncated ORF2 protein. It has been shown that during acute infection, virus-specific T helper cell responses can clear viral infection but lack of its activity leads to chronic infection [MacDonald et al., 2002]. In this study, the HLA typing was not carried out and the frequencies of various HLA alleles among the participants were not determined. So it is not clear which type of HLA alleles among the HEV-recovered individuals had a low or high-affinity HLA binding epitopes against the multiepitope protein.

Li et al. designed a multiepitope construct comprising six low-affinity HIV gag and pol CTL epitopes restricted to HLA-A*0201 and the HIV p24 antigen by bioinformatics analysis. They used cryptic epitope modification to enhance the immunogenicity of low-affinity HLA-A2.1-binding epitpes, and found the multiepitope can induce a strong CD8+ T cell immune responses in mice [Li et al., 2013b]. Jafarpour et al. designed and synthesized a multiepitope construct encoding conserved and immunogenic epitopes from HIV-1 tat, pol, gag, and env proteins by bioinformatics analyses as a new DNA vaccine candidate. The designed multiepitope plasmid was able to induce proper immune responces in BALB/c mice [Jafarpour et al., 2014]. Li et al. designed a multiepitope construct encoding T and B lymphocyte epitopes from latent membrane protein 2 (LMP2) of Epstein–Barr virus (EBV) based on mammalian codon usage. The recombinant plasmid pcDNA3.1+/EBV-LMP2 multiepitope was constructed and transfected into COS-7 cells. This multiepitope plasmid DNA was able to stimulate strong cellular and humoral immunity in mice [Li et al., 2013a].

In the present study, high level of specific IFN-γ and IL-12 responses (T helper type 1 cytokines) and low level of IL-10 and very low level of IL-4 responses (T helper type 2 cytokines) against high density multiepitope protein and truncated ORF2 protein were observed among the HEV-recovered individuals. Thus, both proteins can be considered as promising vaccine candidate against HEV infection. Hashish et al. designed a multiepitope fusion protein compromising two B cell and T cell epitopes from the E2 glycoprotein of bovine viral diarrhea virus (BVDV), and also K99 major subunit FanC and STa toxoid of Enterotoxigenic *Escherichia coli* (ETEC). In their study, FanC-STa-E2 gene construction was cloned into the expression vector pET28 and transformed into *E. coli* BL21 cells. FanC-STa-E2 fusion protein was well expressed by IPTG-induction (1 mM) at 37°C for 4 hr and purified by Ni-NTA agarose using His-tag. This multiepitope fusion protein could develop neutralizing antibodies against ETEC and BVDV in vitro [Hashish et al., 2013].

Beside vaccine development, multiepitope technology can be used for diagnosis. Several studies have

been performed on multiepitopes proteins for development of diagnostic kit. The results of these studies showed that the use of multiepitope proteins can increase the sensitivity and specificity of the tests, since this kind of proteins has great efficiency to expose conserved and immunodominant epitopes [Dipti et al., 2006; de Souza et al., 2013]. de Souza et al. designed a high density multiepitope protein containing three copies of four conserved epitopes from hepatitis B core protein (HBcAg), and developed an ELISA using this recombinant protein. The developed ELISA was useful for hepatitis B diagnosis [de Souza et al., 2013]. Dipti et al. designed a novel recombinant multiepitope protein comprising six conserved and immunodominant epitopes from hepatitis C virus, and developed an anti-HCV diagnostic kit using this protein. The multiepitope protein was able to detect anti-HCV antibodies in human plasma with high degree of sensitivity and specificity [Dipti et al., 2006].

Although several microorganisms and cell lines are currently used for expression of foreign genes, but still *E. coli* are routinely used for expression of target proteins [Vincentelli and Romier, 2013]. Fast cultivation, well-known genetics and high-level expression capabilities with high purity are some other advantages of *E. coli* expression system [Fakruddin et al., 2013]. Despite all these advantages, expression of a foreign gene in *E. coli* may be hampered by the presence of rare codons and high GC contents within the gene-coding region which result in decrease of the expression level or production of insoluble and nonfunctional proteins [Mondal et al., 2013; Elena et al., 2014]. In the present study, *E. coli* BL21 cell was used for expression of the high density multiepitope protein. The target genes were cloned in to the pET30a+ plasmid and expressed by IPTG-induction under control of strong bacteriophage T7 promoter and T7 RNA polymerase of the *E. coli* BL21 cell. The IPTG-induction is economically desirable method and increases protein production [Mondal et al., 2013]. Previous studies have reported high yield protein purification by Ni2+ chelate affinity chromatography using His-tag [Glynou et al., 2003; Farshadpour et al., 2014]. The application of affinity tags is simple, rapid, cost effective and also efficient for purification of proteins, and also has no effect on the structure, folding and function of the protein [Glynou et al., 2003; Carson et al., 2007]. In the present study, His-tag was used for the purification. The results of this study show that *E. coli* is a good system for expression of recombinant proteins with high yield.

In conclusion, a high density multiepitope protein capable of induction of T helper type 1 responses was successfully produced and evaluated in vitro. The results of this study provide much information for the design of HEV multiepitope vaccine. However, further works will be needed to evaluate protective immune response of the high density multiepitope protein in vivo.

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