

## Human $\beta$ hsp90 as adjuvant in HCV Recombinant vaccine

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### ABSTRACT

There are more than 350 million individuals with hepatitis C in the world. One of the important problems in vaccine project is development of effective and suitable adjuvant in human vaccines. At present research we applied human  $\beta$ Hsp90 protein as an adjuvant in recombinant HCV vaccine design. The thermal vector of pGP1-2 was used for human heat shock protein 90 expression. This protein injected to BalbC mice as an adjuvant together with recombinant protein of HCV core. The combination of these proteins was used and we evaluated the humoral and cellular immunity and the cytokine secretion of inguinal and popliteal lymph nodes lymphocytes were analyzed in vitro and ex vivo conditions. So the combination of Core protein together with hsp90 induced total IgG and IgG2a secretion. The spleen lymphocytes proliferation were increased equal to serum IgG2a level that was constant in second time bleeding with significant different to complexes with freund's adjuvant. At first IL-4 and IL-5 cytokines were increased, after one week it decreased. Production of IL-4 showed there was no hypersensitivity reaction after vaccine injection.

**Keywords:** Hsp90; core; lymphocyte; IgG; pETDuet-1 vector

### INTRODUCTION

It was estimated that about 350 million with hepatitis C virus (HCV) [1, 2]. This virus are the most common cause of liver diseases. It can be transmitted parenterally, sexually and prenatally [1]. Search for new adjuvant, able to selectively trigger protective antibody production [3], is one of the important approaches in this study. However peptides bound to heat shock proteins (HSPs) of microbial or mammalian origin have been shown to elicit potent antigen-specific immunity. Also some of them, such as hsp60, hsp70, hsp90 and gp96 act as danger signaling molecules [4]. The ability of HSPs to facilitate the cross-presentation of MHC class I restricted epitopes and to prime CD8 cell effectors responses has been well established [5]. CD4+ T cells are important in shaping adaptive immune

effectors like B cells and CD8+ T cells. The difference between spontaneous resolving and chronic persistence seems to lie on the quality of the CD4+ T cell response [6]. The main purpose of this study was to elucidate immune responses induction, associated with formulation of the antigen and human Hsp90 as adjuvant.

### MATERIALS AND METHODS

#### *The Cells, bacterial strains, plasmids and laboratory animals:*

The THP-1 cell line and BalbC mice were obtained from the Pasteur Institute of Iran. Escherichia coli strains Nova Blue and DH5 $\alpha$  were purchased from Invitrogen, USA. pTZ57R (Fermentas, Germany), pGPhsp90 (constructed in our before study) and pETDuet-1 were plasmids which were used in this study.

**Cloning and Expression of HCV core protein:**

The plasmids pCgemex-1[7] was used to generate the HCV core PCR product by using below primers:

CoreF5'CATATGAGCACACTTCCAAAACCCC3', CoreR 5'CTCGAGCGCGGAGGCTGGTGTGTAAG3'.

To express interested protein, the PCR product was inserted into pETDuet-1 expression vector after digestion with NdeI, XhoI restriction enzymes. Protein expression induction of HCV core was carried out by 1 mM IPTG at 37° C. Hsp90 protein expression was induced by 42°C after 5 hours. Confirmation of these proteins were carried out by western blotting and gel diffusion with mixed patients sera and monoclonal antibodies of tag peptides[8].

**Study of biological activity of HCV core protein**

Macrophages and dendritic cells are the major sources of IL-12 produced during an inflammatory response. Thus the suppression of IL-12 by HCV core might be due to a direct interference with Mφ/DC activation by these antigens [9]. So at first THP-1 monocyte cell line was inoculated in RPMI medium (10% FBS, 100µg streptomycin, 100unit penicillin). Then the cells were transferred to 24-well plates, 20000 cells in each well. After 6h they were induced to macrophages by 250 ng PMA. These mature cells were incubated for 24h at 37 ° C in 5% Co2. To induce the IL-12 production by Mφs, 250 ng/ml Escherichia coli LPS was added to half of the wells. After 24h ELISA assay (Duoset ELISA kit, R&D) was carried out for study of IL-12 production changes.

**Immunization of mice with HCV core protein**

After confirmation of the biological activity of produced protein, mice were immunized with 10µg protein with either equal volumes of freund's complete adjuvant (sigma, USA) or in mixture with 5 µg purified rhsp90 [8] by subcutaneous injection into the footpads. Control mice were immunized with FCA, rhsp90, LPS and PBS alone. The injection volume was adjusted to 50 µl. The mice were grouped as below: 1- HCC + FA, 2- HCC+hsp, 3- Subcutaneously HSP90, 4- Subcutaneously PBS, 5- Subcutaneously FCA. The mice were injected three times with 10 days intervals. Determination of antibody production in serum and supernatant of

cultured lymphocytes of lymph nodes: Sera of immunized mice were collected on 7 and 14 days after immunization. Preimmune sera were used as controls. The total IgG, IgG2a antibodies were detected using mouse IgG2a and IgG ELISA quantitation kit (Bethyl, TX). The lymphocytes of popliteal and inguinal lymph nodes lymphocytes in injected mice were prepared 14 days after immunization using ficoll 700(Gibco,UK). The lymphocytes layer were suspended in RPMI (Gibco, UK) supplemented with 10% fetal calf serum, 100IU/ml penicillin and 100µg/ml streptomycin. Then for priming the lymphocytes 10µg of protein, PBS, PHA (Gibco, UK) as controls were further added to the culture medium. The cells were incubated for 24 hours at 37° C in a humidified atmosphere with 5% CO<sub>2</sub>. Supernatants were collected after 24 and 48 hours.

**Cytokines assay**

The IL-5, IL-4 and IFN $\gamma$  cytokines were analyzed in the supernatants of the lymphocyte cultures by mouse cytoset ELISA kits (invitrogen, Germany) regarding manufacture protocol.

**Statistical analysis**

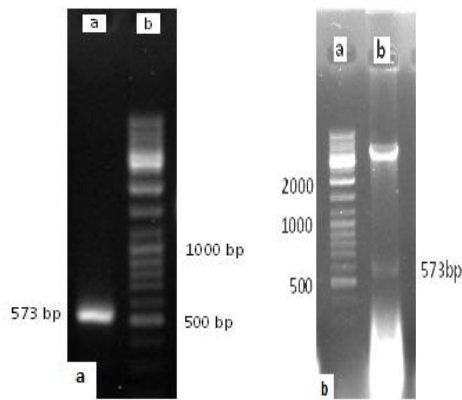
Standard deviation was calculated using SPSS16 software and ANOVA analysis.

**RESULTS**

Sub cloning and expression of HCV core fragment was carried out which results were shown in figures 1 and 2.

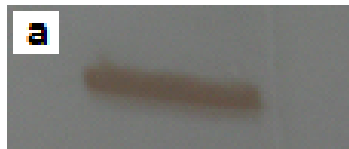
**Serum Antibody analysis by ELISA test**

The serum antibodies of mice were analyzed on 7 and 14 days after injection. Pre-immune sera were used as controls. According to below curves, antibody against recombinant core protein in combination with Freund's adjuvant in secondary response has been decreased. Also serum IgG2a level in group which hsp90protein used as adjuvant was significant (Pv<0.005). So lymphocytes proliferation was depend on antigen effect and their activity was shown with IgG2a production in serum (Fig.3).



**Figure 1: a)** 573bp HCV Core PCR product (a), 100bp DNA Ladder (b)

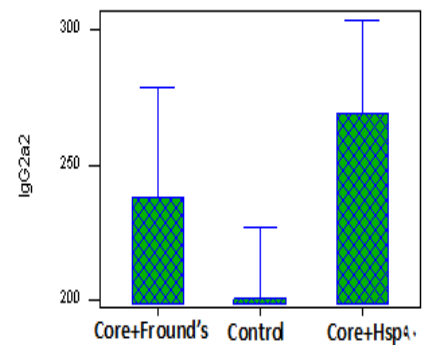
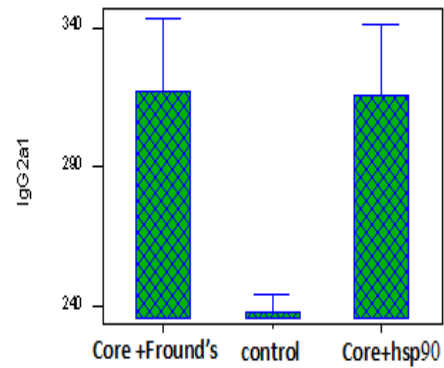
**b)** Cloning confirmation of HCV core fragment by restriction analysis (NdeI, XhoI)



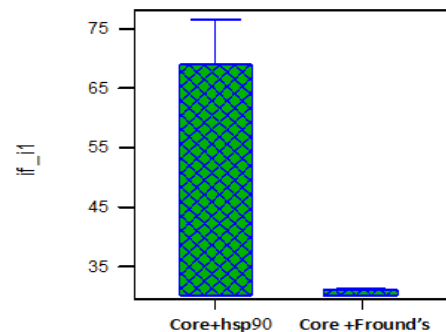
**Figure 2:** confirmation of expressed Core protein with S tag monoclonal Ab

**Evaluation of IL-4, IL-5 and IFN- $\gamma$  cytokines in lymphocytes culture supernatant**

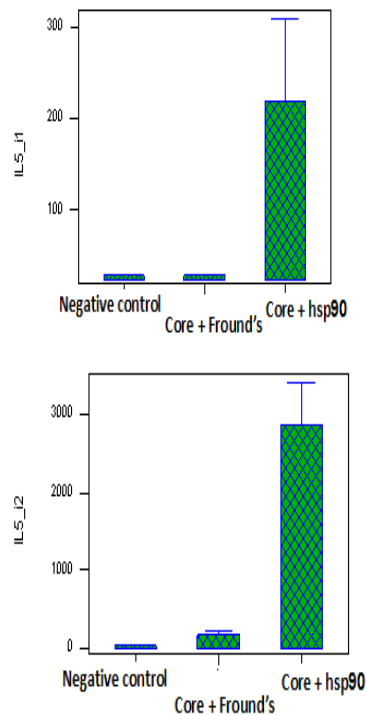
In group immunized with combined protein with hsp90 adjuvant compared with Freund's, IL-4 production is significant. According to below curve reduction of IL-4 production in second step showed that there was not any allergic reaction against of vaccine injection. Hsp90 in combination with HCV core protein had positive effect on inguinal and popliteal lymph nodes lymphocytes for IL-5 production and stability of it in second step. IFN- $\gamma$  production after hsp90 injection as adjuvant and stability of it in inguinal lymphocytes culture were shown in below curve (Fig. 4, 5). After 48 hours, Hsp90 was an effective adjuvant in each of three organs' lymphocytes and it recorded by IL-5 and IgG2a production ( $P < 0.05$ ). It showed excellent systemic immunity responses.



**Figure 3:** Comparison of adjuvant effect of Hsp90 and Freund's in IgG total and IgG2a production against of Core Ag after 24(1) and 48(2) hours



**Figure 4:** Comparison between IFN- $\gamma$  (pg/ml) in groups with combination of Core protein and Hsp90 or Freund's adjuvant after 24h



**Figure 6:** Comparison between Core protein mixed with hsp90 or freund's adjuvant injected groups for IL-5 production (pg/ml) after 24h (i1) and 48h (i2)

## DISCUSSION

Viral hepatitis infections associate with 106 annual mortality rate in human population, globally [10]. In spite of vaccination in the different areas there are several reports about patients who got vaccine before. Also there is not efficient vaccine against of hepatitis C and one of the problems in vaccine project is development of effective and suitable adjuvant in human vaccines. The main purpose of this study was antibody production and cytokine variation against recombinant core protein mixed with rhsp90 as adjuvant compared with freund's. There are many reports implying that chaperones may act as immunological adjuvant and could trigger potent cellular immune responses against intracellular pathogenic agents. Among broad family of chaperone proteins, beta subunit of human heat shock protein 90 was considered as adjuvant counterpart of the vaccine. HCV core protein was considered as immunogenic counterparts of the vaccine. Serum antibodies

against HCV core protein epitopes (residues 7-21, 31-45, 49-63, 99-113) have been demonstrated in HCV patients [11]. Due to contribution of all amino acid residues to form exact conformational epitopes, total of 191 amino acids were included in the vaccine preparation. Epitope specific T cell clones, produced through application of the vaccine, are able to recognize and act against virus infected cells. In 2004, Doody et al, have revealed that a glycoprotein of 96 KD contributes to refolding of MHC-I and MHC-II molecules and to appropriate presenting of antigens and also results in differentiation of T CD8+ cells to effectors ones. To escape unwanted derivative and destructive outcomes of freund's adjuvant, many scientists are seeking for an alternative strong and polarizing adjuvant appropriate for use with viral vaccines [12]. Several studies for evaluation of immune responses elicited by HCV core protein have been done, including, identification of core protein specific cytotoxic T lymphocytes [13]. Also Wen Li et al, in their study on 2006, Canada, have demonstrated high expression of pro inflammatory cytokines, stimulation of T cells and presence of Dendritic cells of expressing HCV-core Ags or NS3 proteins [14]. According to the patent, number WO/2005/025614, injecting dose of the adjuvant to simultaneous application was one fifteenth of each of recombinant antigen.

Our findings showed that, as a potent immunostimulant, freund's compound could be replaced by HSP protein in the future vaccine formulations. Preliminary increased levels of IL-4 imply proper humoral immune stimulation in the first encounter and following decrease of the cytokine means no hypersensitivity reactions could occur. Increased IL-5 levels in the study appropriately are related with the antibody levels, also increased concentration of the cytokine in inguinal lymphocytes culture are related to enough stimulation of the immune system achieved by vaccination.

In general, stimulatory effects of Freund's adjuvant to increase production of antibodies were seen, but some instability of the produced antibody in second sampling was demonstrated.

**CONCLUSION**

So often stimulation of cells through application of HSP90 together with antigen occurs only locally and leading forward to IFN $\gamma$ , IL-5 and IgG2a production, and no accordance of hypersensitivity reactions, but in the case of Freund's adjuvants also some of vaccinated mice showed hypersensitivity reactions.

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