Liposome-Based HCV Vaccine Enhances Protective Cellular Immunity and IFN-γ Secretion in Mice

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Hepatitis C virus (HCV) is a principal cause of acute and chronic hepatitis (Ebeid et al., 2009), placing infected persons at risk for liver cirrhosis, hepatocellular carcinoma and extra-hepatic complications, that develop over decades following onset of infection (Ghany et al., 2009). World Health Organization estimates that 3% of the world population is chronically infected with HCV (Lavanchy et al., 2011). Egypt has the highest prevalence of HCV in the world. According to the Egyptians demographic health survey in 2009, the prevalence rate of HCV antibody positivity was 14.7%, and the number of Egyptians estimated to be chronically infected, was 9.8% (El-Zanaty et al., 2009).

Away from mechanisms to escape innate immunity, HCV also employs strategies to escape host adaptive immunity to be protected against antibody neutralization. HCV-specific T lymphocytes are detectable 5 to 9 weeks after infection which coincides with the onset of hepatitis (Zeisel et al., 2009). Both CD4+ and CD8+ T cells have been shown to play major roles in the outcome of HCV infection. A strong HCV-specific CD8+ T-cell response, is correlated with the control of viremia during the acute phase (Strader et al., 2004). CD8+ T cells inhibit HCV replication, which is highly dependent on sufficient CD4+ T-cell cooperation. Indeed, vigorous peripheral and intrahepatic virus-specific T cell responses have been described in patients who recover from HCV infection and a functionally impaired T cell response was reported in patients who fail to clear the virus (Rehermann et al., 2009). Viral escape, lack of CD4+ T-cell cooperation, and regulatory T cell suppression are all factors that potentially...
Interferon gamma (IFN-γ) is the sole type II IFN. It is structurally unrelated to type I IFNs, bind to a different receptor, and is encoded by a separate chromosomal locus. T lymphocytes are the major source of IFN-γ in the adaptive immune response. IFN-γ may inhibit HCV virion production by an effect on viral RNA and protein synthesis. It enhances immune lysis of HCV infected cells and inhibits hepatic fibrosis by an effect on transforming growth factor beta (Cimini et al., 2012). It has also direct non-cytolytic antiviral activity and can inhibit HCV replication in vitro (Lanford et al., 2003).

Pegylated interferon and ribavirin therapy, although beneficial in about half of treated patients, are expensive and associated with momentous side effects. In this clinical context, there is an urgent need for the development of a therapeutic and/or prophylactic HCV vaccine (Callendret et al., 2011). To date, there is no effective vaccine against HCV infection. Efforts to develop an HCV vaccine are complicated by the extensive genetic and antigenic diversity among HCV strains and the absence of a robust immunity after natural infection (Kuntzen et al., 2008). HCV persists in the presence of circulating antibodies and it has been speculated that this recounts to the highly mutable nature of this RNA virus and the continual emergence of neutralization-resistant strains. However, the persistence of HCV in the presence of anti-HCV antibodies cannot be fully explained by high variability alone (Logvinoff et al., 2004).

Liposomes are vesicles made up of one or more concentric lipid bilayers alternating with aqueous spaces. The lipid components are usually phospholipids or other amphiphiles such as nonionic surfactants, often supplemented with cholesterol and other charged lipids (Gregoriadis et al., 1990). Liposomes are produced from natural or synthetic compounds at high purity, owing to their ability to entrap water- and lipid-soluble molecules in their aqueous and lipid phases, respectively, liposomes have been used as a delivery system for a great variety of pharmacologically active agents in therapeutics and have been safely used in humans in several clinical studies, such as in anti-cancer treatment (Butts et al., 2009).

The present study utilizes the availability of C57Bl/6J mice as a preclinical animal model and investigated the requirement of a viral core protein (CD4+ T cell epitope) for the efficient priming of a CD8+ T cell response. It concentrated on the capacity of such vaccine formulations to stimulate cellular proliferation as well as IFN-γ secretion.

Materials and Methods

Experimental Animals

The study was conducted on a total of 95 female inbred (C57Bl/6J) mice (15 - 20 gm weight) provided by Theodor Bilharz Research Institute, Cairo. During the entire study, mice were kept at the animal care facilities of the Department of Immunology and Allergy, Medical Research Institute, University of Alexandria, and experiments were conducted according to the international guidelines for animal experimentation. They were divided into five groups:

- Group I: included 30 mice injected with purified recombinant HCV core antigen (of genotype 1a) carried on liposomes.
- Group II: included 30 mice injected with purified HCV core antigen and complete Freund’s adjuvant (CFA).
- Group III: included 15 mice injected with empty liposomes.
- Group IV: included 15 mice injected with core Ag (CA).
- Group V (Control): included 5 mice injected with saline.
Preparation of Liposomes

Liposomes were prepared by the thin film hydration method (Ludewig et al., 2000): Three mg phosphatidylcholine (Sigma) and cholesterol (3/1, w/w) were dissolved in 10 ml of chloroform and ethanol mixture (3/7, v/v) (model laboratoriums-Technik AG.CH-9230-Switzerland). Extraction of solvent from the nano-droplets was achieved by evaporation at 55°C in the rotary evaporator under reduced pressure (200 mmHg) for 15 minutes till a thin film obtained in the inner wall of the flask, leading to the formation of nanoparticles by precipitation of the macromolecules. The dry lipid was hydrated with 10 ml phosphate buffer (pH=7.4) followed by shaking for 15 minutes at low speed at 55°C, then 15 minute hand shaking at room temperature to obtain a lipid suspension.

Incorporation of HCV Epitope (HCV core protein) into Lipid Vesicles

Liposomes loaded with HCV core antigen were prepared as the following (Schwendener et al., 2010): 100 µg of purified recombinant HCV core antigen of purity greater than 95% (LKT Laboratories Inc., LKT-R2711-M001) and the vessel were vigorously agitated on a rotary mixer. Recombinant HCV Core encodes the immunodominant sequences of the HCV nucleocapsid p21 protein, amino acids 2-119; it was purified by proprietary chromatographic techniques. The produced multi-lamellar vesicles (MLVS) were then bath-sonicated using Bransonic Ultrasonic B-220 water bath-sonicator at 55°C for 15 minutes to produce uni-lamellar liposomes. The prepared liposomal suspension was centrifuged with ultracentrifuge (Sigma 3K 30 laboratory centrifuges) at 10,000 rpm at 4°C for 30 minutes. The resulted supernatant was analyzed for antigen content by measuring the absorbance at 695 nm which represents antigen free concentration. The antigen uptake by the liposomes (encapsulation efficiency) was determined by calculating the difference between the initial and residual amounts of antigen in the preparing solution. The encapsulation efficiency (EE) was estimated as follow (AT is the total antigen added to the system through liposome preparation, and AF is the free antigen in the supernatant):

\[ \text{EE}\% = \frac{(\text{AT} - \text{AF})}{\text{AT}} \times 100 \]

Characterization of Liposomes

- Transmitting electron microscopy (TEM)

The physical size and morphology of the prepared liposomes; empty and loaded with antigen were determined by transmission electron microscope (JEOL-100 CX) (Li et al., 2011). The samples prior to examination were bath-sonicated at 55°C for 15 minutes. Samples (10 µL) were placed on 400-mesh copper grids coated with carbon film, waiting for 2 minutes to allow adsorption of the liposomes on the carbon film. After one minute, excess solution was removed; the grids were stained with 2% uranyl acetate in water for 2 minutes, and allowed to dry. Then samples were examined by the electron microscope. According to the transmission electron microscope images, the discrete liposomes were spherical and their round shapes maintained with homogeneous structures.

- Scanning electron microscopy (SEM)

The morphology and surface appearance of liposomes; empty and loaded with antigen, were determined by scanning electron microscopy JEOL (JSM-5300). One drop of the particles suspension was placed on a gold-coated plate and maintained at least for 12 hours at room temperature in desiccators for complete dryness of the sample. The dry samples were coated with a thin gold layer using coater prior to the microscopic examination.

Immunization Protocol

Experimental groups (I, II and IV) received 130 ng of purified HCV core antigen either with liposomes (gp I), with CFA (gp II) or alone (gp IV). Group III received empty liposomes. Control group received only normal saline. Injection was carried out for all mice subcutaneously (S.C.) at the abdominal region in a total volume of 100 µl for 3 times at weekly intervals (Engler et al., 2004). Complete Freund’s adjuvant (CFA) is a mixture of paraffin oil and surfactant with heat-killed Mycobacterium tuberculosis in which aqueous antigen solutions are emulsified (Freund 1956). Although CFA is unacceptable for human use, studies on the mode of action of this potent adjuvant can provide useful lessons for vaccine design (Look et al., 2010).

Preparation of Splenocytes Suspension

Spleens were excised from all mice under study one week after the last injection. This was done under strictly aseptic conditions according to the method originally employed by Coligan et al. (1994). Splenocytes were squeezed out of the splenic capsule in petri dishes containing phosphate buffer saline (PBS) of pH=7.2. Cell suspensions were then transferred from the petri dishes into sterile 15 ml falcon tubes and centrifuged 2000 rpm for 10 minutes. Contaminating RBCs were lysed by an ammonium chloride solution (0.87%). At the end of the ammonium chloride treatment, splenocytes were washed twice in culture medium and finally resuspended in tissue complete
culture medium (RPMI-1640) (Biochrom KG Berline) supplemented with fetal calf serum (FCS) (Seromed, Germany), 2 mM L-glutamine (Gibco, Scotland), Penicillin (100 IU/ml) and Streptomycin (100µg/ml) (Seromed, Germany).

Viability and Purity Testing

Viability of the separated cells was tested by dye exclusion technique which is based on the impermeability of viable cells to Trypan blue 0.4 % (0.4 gm in 100 ml redistilled water and stored at room temperature). 120 µl of the diluted suspension was added to 120 µl of Trypan blue solution, left for 5 minutes and examined microscopically using a haemocytometer. Four white squares were counted for viable cells. The number of viable cells per ml was calculated according to the following equation:

\[ \text{No. of viable splenocytes/ml} = \frac{\text{No. of splenocytes counted in 4 white squares} \times \text{dilution factor} \times 10^7}{4} \]

Cell Cultures

Splenocytes (lymphocytes) adjusted to 2×10^6 cells/ml were maintained in a short-term (48 hours) culture for assessment of cell proliferation by MTT and estimation of IFN-γ levels. Details of the tissue culture protocol were adopted from the standard method developed by Davis et al., (1995). Triplicate wells containing spleen cells were used, 100 µl of splenocytes suspension (2 x 10^6/ml) were added to all wells corresponding to each of the assay animal. 100 µl of the supplemented RPMI-1640 tissue culture medium were dispensed in 96 wells microtitre flat-bottomed tissue culture plate. Two sets of three wells each; were then considered. Volumes of 10 µl (10 µg/ml) HCV core antigen peptide and 10 µl concavalin A (con A) (plant mitogen, known for its ability to stimulate mouse T-cell subsets) (Dwyer et al., 1981) were added only to the first set. The other set was left with only con A stimulation as a control. The plate containing the specifically re-stimulated and the non-stimulated spleen cells from immunized mice was then incubated for 48 hours (5% CO2) at 37°C.

MTT Assay

After finishing the 48 hours of the cell culture, 10 µl of MTT reagent (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) (Sigma, USA) were added to each well containing splenocytes cultured in 96 flat-bottomed microtitre plate as previously described. The cells were incubated for four hours at 37°C in a CO2 incubator. After incubation, the formazan produced in the cells appeared as dark crystals in the bottom of the wells. 100µl of crystal dissolving solution were added to each well and incubated for 10 minute. This solution dissolves the formazan crystals, producing a purple solution. The solubilized formazan product was spectrophotometrically quantified; so microplates were evaluated with the use of an ELISA reader at 570 nm with a reference wavelength of >650 nm. An increase in number of living cells results in an increase in the total metabolic activity in the sample. This increase directly correlates to the amount of purple formazan crystals formed. The absorbance was measured for each sample and stimulation indices were calculated as following:

\[ \text{Stimulation index (S.I)} = \frac{\text{Absorbance with stimulation}}{\text{Absorbance without stimulation}} \]

IFN-γ Levels

IFN-γ level was estimated using commercial enzyme linked immunosorbent assay (Quantikine R&D system, ELISA kit, USA) according to manufacturer recommendation. Monoclonal antibody specific for mouse IFN-γ has been pre-coated onto a microplate. 50 µL of standards, control, and culture supernatant samples were pipetted into the wells and any mouse IFN-γ present is bound by the immobilized antibody. An enzyme-linked polyclonal antibody specific for mouse IFN-γ was added to the wells in a volume of 100 µL per well, then 100 µL of substrate solution. The enzyme reaction yields a blue product that turns yellow when 100 µL of Stop Solution were added to each well. The intensity of the color measured was in proportion to the amount of mouse IFN-γ bound in the initial step. Using a microplate reader set to 450 nm, the optical density (OD) was measured taking blank well as zero. According to standards’ concentration and the corresponding OD values, were calculated out the standard curve linear regression equation, and then applied the OD values of the sample on the regression equation to calculate the corresponding sample’s concentration. The sample values are then read off the standard curve.

Statistical Analysis

All data were presented as mean±SD (standard deviation of mean) they were compared with the tabulated probability value (P value) as the 0.05 level using SPSS statistical package (SPSS Inc., Chicago, IL). P value was considered significant if it is ≤0.05. The following statistical tests were used: Mann-Whitney Rank-Sum test, Student t-test, Paired t-test, Wilcoxon signed ranks test and linear correlation coefficient “r” to examine the relationship between different parameters.
Results

Experimental Animals

The first group of mice which consisted of 30 mice received 130 ng of purified HCV core antigen carried on liposomes at weekly intervals as described before. The second group (30 mice) was immunized with the purified core HCV antigen but with a complete Freund’s adjuvant. Three more groups were injected with either empty liposomes (15 mice) or purified HCV CA alone (15 mice) or lastly saline (5 mice) and used as a comparative control groups. Injections were carried for three times at weekly intervals and the animals were followed for two weeks after the last vaccination (Engler et al., 2004).

Purification and Characterization of Reconstituted Vesicles

Liposomes Morphology Studies

The TEM image analysis of empty liposomes showed that, the particles were spherical in shape with size range from 14.9 to 103 nm with a well intact lipid film forming the edge of each nanosphere, (Figure 1). The SEM image revealed no particle aggregation, figure (2). TEM ensured that, the liposomes encapsulated HCV core antigen were larger in size than the empty liposomes, sizes were not uniform and varied in the range from 120 nm to 177 nm, (Figure 3).

Determination of Antigen Entrapment Efficiency

A commercial total protein determination kit (Biosciences, LLC, USA) was used to measure the total vaccine concentration (HCV core antigen) and the free vaccine in the supernatant after centrifugation. The percentage of vaccine entrapment estimated in all liposomal formulations was 87% indicating that these nanoparticles have potential for use as vaccine delivery agents.

Figure 1. Transmission Electron Microscope image of empty liposomes (magnification : 10000 x)

Figure 2. Scanning Electron Microscope image demonstrating empty liposomes with almost spherical shapes, not sticking to each other.
Figure 3. Transmission electron microscope image of HCV core antigen encapsulated liposomes (magnification: 10000 x).

Cellular proliferation results (MTT)

Spontaneous Lymphocyte Proliferation in Cultures

Results of assessment of spontaneous splenocytes proliferation using MTT are summarized in table 1. Statistical analysis showed that, without specific stimulation, mice immunized with liposomes and core Ag had the highest proliferation (mean=459.47 OD units) with a highly significant increase compared to the other groups (empty liposomes, $P < 0.001$) (CFA injected group, $P = 0.012$) and (saline injected group, $P < 0.001$). The second highest spontaneous cellular proliferation was noticed in group II that were injected with core Ag and CFA (mean= 371.40 OD units, $P = 0.018$, when compared to saline injected group). Nevertheless, statistical analysis showed a significant increase in proliferation in the (core + liposome) group compared to the second group (core + CFA) ($P < 0.001$).

Group injected with core Ag alone showed a non-significant difference from the control group (saline) $P = 0.126$.

Stimulated Lymphocyte Proliferation in Cultures and Stimulation Index

Proliferation of stimulated splenocytes in these short term cultures revealed also a highly significant elevation in mice immunized with liposomes and core Ag (mean=661.1) ($P <0.001$) with a stimulation index mean = 1.47 ($P <0.001$). Group II showed a stimulation index of 1.29 ($P = 0.003$) with a significant difference from group I ($P = 0.001$), however, group II immunized with core Ag and CFA did not show a significant difference from group IV which were injected with core Ag alone $P =0.665$. A comparison between cellular proliferation before and after stimulation as well as stimulation indices are summarized in table 1.
Table 1. Cellular proliferation (MTT) assay results in mice immunized with liposomal-based HCV vaccine

<table>
<thead>
<tr>
<th>Group</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunization</td>
<td>Liposomes with core Ag</td>
<td>CFA with core Ag</td>
<td>Empty liposomes</td>
<td>CA</td>
<td>Saline</td>
</tr>
<tr>
<td>Number of mice</td>
<td>30</td>
<td>30</td>
<td>15</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Spontaneous proliferation mean± SD</td>
<td>459.47 ± 119.97</td>
<td>371.4 ± 144.74</td>
<td>307 ± 90.57</td>
<td>255.8 ± 56.82</td>
<td>206 ± 59.41</td>
</tr>
<tr>
<td>P1</td>
<td>0.012*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>P2</td>
<td>0.018*</td>
<td>0.029*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>P3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.027*</td>
</tr>
<tr>
<td>Stimulated proliferation range</td>
<td>395-921</td>
<td>210- 704</td>
<td>179- 526</td>
<td>250-390</td>
<td>151-288</td>
</tr>
<tr>
<td>Stimulated proliferation mean± SD</td>
<td>661.1±148.6</td>
<td>454.17±166.17</td>
<td>361.47±94.33</td>
<td>314.27±38.82</td>
<td>219±63.24</td>
</tr>
<tr>
<td>P1</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>P2</td>
<td>0.002*</td>
<td>0.008*</td>
<td>0.005*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td></td>
<td></td>
<td>0.010*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stimulation Index</td>
<td>1.47</td>
<td>1.29</td>
<td>1.19</td>
<td>1.26</td>
<td>1.06</td>
</tr>
<tr>
<td>P1</td>
<td>0.001*</td>
<td>0.001*</td>
<td>&lt;0.001*</td>
<td>0.002*</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>0.003*</td>
<td>0.061</td>
<td>0.008*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

P: Mann Whitney test comparing between group I vs. each other groups
P2: control vs. each other group
P3: group II vs. group IV.
*: P ≤ 0.05 is significant. NS= not significant
IFN-γ Level

Spontaneous Secretion of IFN-γ in Culture Supernatants

Levels of IFN-γ secreted spontaneously in culture supernatants of cultured splenocytes for 48 hours measured by ELISA and expressed in pg/L unit, showed also a highly significant increase in the group of mice which received the core antigen in liposomes (mean ± S.D = 56.30 ± 8.71) when compared to all other groups (p< 0.001). This was followed unpredictably by the empty liposomes group (group III), mean = 43.79 ± 9.41, but with a highly significant difference from group I (p=0.001). The lowest level of spontaneously secreted IFN-γ was found in the control group (1.78 ± 0.21) pg/L. Comparison between different levels detected in all studied groups are presented in table 2.

IFN-γ Levels in Culture Supernatants after Stimulation

IFN-γ secreted in culture supernatants of cultured splenocytes after they were stimulated in short term cultures, measured by ELISA and expressed in pg/L unit, showed an elevated level, which was highly significant in group I (mean ± S.D = 68.32 ±18.47. The maximum secreted level was 117.52 pg/L and this level wasn’t detected in any other group. Control group showed a significantly very low level of the cytokine (mean=6.19± 0.71) when compared to all other groups (P < 0.001). The other three groups showed a nearly similar secretion of IFN-γ but with a significant rise in group II (containing the adjuvant) than group IV (without CFA) (P =0.048) (table 2). A comparison of different studied groups according to IFN-γ with and without stimulation is shown in figure 4.

Figure 4. IFN-γ levels (µg/ml) in cell culture supernatant with and without stimulation in different studied groups of vaccinated mice.
Table 2. Interferon gamma level (ELISA) in mice immunized with liposomal-based HCV vaccine

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunization</th>
<th>Liposomes with core Ag</th>
<th>CFA with core Ag</th>
<th>Empty liposomes</th>
<th>CA</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of mice</td>
<td>30</td>
<td>30</td>
<td>15</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Spontaneous IFN-γ range</td>
<td>33.77 – 67.70</td>
<td>22.17 – 58.68</td>
<td>27.33 – 55.66</td>
<td>29.90 – 44.08</td>
<td>1.62 – 2.01</td>
</tr>
<tr>
<td></td>
<td>Spontaneous IFN-γ mean± SD</td>
<td>56.30 ± 8.71</td>
<td>39.90 ± 10.22</td>
<td>43.79 ± 9.41</td>
<td>35.34 ± 5.12</td>
<td>1.78 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>&lt;0.001*</td>
<td>0.001*</td>
<td>0.001*</td>
<td></td>
<td>0.041*</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stimulated IFN-γ range</td>
<td>38.27-117.52</td>
<td>33.34-82.73</td>
<td>31.19-60.83</td>
<td>37.20-71.6</td>
<td>5.42-6.71</td>
</tr>
<tr>
<td></td>
<td>Stimulated IFN-γ mean± SD</td>
<td>68.32 ±18.47</td>
<td>57.99 ± 16.80</td>
<td>48.02± 9.34</td>
<td>46.24±8.61</td>
<td>6.19±0.71</td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>0.041*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
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<td>&lt;0.001*</td>
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<tr>
<td></td>
<td>P2</td>
<td>&lt;0.001*</td>
<td>0.001*</td>
<td>0.001*</td>
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<tr>
<td></td>
<td>P3</td>
<td></td>
<td></td>
<td></td>
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<td>0.048*</td>
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</tbody>
</table>

P1: P value for Mann Whitney test comparing between group I vs each other groups
P2: P value comparing between control vs each other group
P3: P value comparing between group II vs group IV.

Group I: mice injected with purified HCV core antigen carried on liposomes.
Group II: mice injected with purified HCV core antigen and complete Freund’s adjuvant (CFA).
Group III: mice injected with empty liposomes. Group IV: mice injected with core Ag (CA).
Group V: mice injected with saline (control group). *: Statistically significant at $P \leq 0.05$

Correlation Studies

Correlation studies were done among all studied parameters in overall groups. Results are summarized in figures 5 - 10.

A positive correlation was found between splenocytes proliferation using MTT assay without stimulation and level of IFN-$\gamma$ with and without stimulation ($r=0.606^*$, $P<0.001$) and ($r=0.680^*$, $P<0.001$) respectively.

A positive correlation was also found between splenocytes proliferation with stimulation and level of IFN-$\gamma$ with and without stimulation ($r=0.645^*$, $P<0.001$) and ($r=0.758^*$, $P<0.001$) respectively.

Stimulation index showed a positive correlation with level of IFN-$\gamma$ without stimulation ($r=0.336^*$, $P=0.001$) as well as with stimulation ($r=0.237^*$, $P=0.021$).

A positive correlation was found between levels of splenocytes proliferation using MTT assay without stimulation and level of IFN-$\gamma$ without stimulation ($r=0.440^*$, $P<0.15$).

A positive correlation was also found between levels of splenocytes proliferation using MTT assay without stimulation and level of IFN-$\gamma$ with stimulation ($r=0.513^*$, $P=0.004$), (table 3).

Finally, splenocytes proliferation with stimulation had a positive correlation with level of IFN-$\gamma$ without stimulation ($r=0.419^*$, $P=0.021$) as well as with stimulation ($r=0.517^*$, $P=0.003$) (table 3).
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Figure 5. Correlation between MTT without stimulation and IFN-γ without stimulation in total groups

Figure 6. Correlation between MTT without stimulation and IFN-γ with stimulation in total groups

Figure 7. Correlation between MTT with stimulation and IFN-γ without stimulation A in total groups

Figure 8. Correlation between MTT with stimulation A and IFN-γ with stimulation A in total groups

Figure 9. Correlation between stimulation index and IFN-γ without stimulation A in total groups

Figure 10. Correlation between stimulation index and IFN-γ with stimulation in total groups
Discussion

Countless attempts have been performed to develop a vaccine against HCV infection. Even though some promising results were achieved, no effort on vaccine expansion has been fruitful. Recent studies showed that epitope vaccines might be a feasible strategy for HCV vaccine designs than the whole HCV virus (Gupte et al., 2012) (Yohoko et al., 2013). HCV core vaccine was shown to mount both humoral and cellular responses against the virus (Jia-Yu et al., 2006). The core protein interacts with numerous cellular proteins and affects host cell functions such as gene transcription, apoptosis and various signaling pathways (Tellinghuisen et al., 2002). In this study, we chose a T cell epitope of HCV core region to design HCV vaccine; it has demonstrated specific CD4+ T cell response against the core protein in many experiments. Vaccination potential of core protein has also been tested through the novel strategy of DC vaccination (Isaguliants et al., 2004). It is hence, one of the prime targets for intervention. Recombinant HCV envelope (E1) and (E2) proteins in experimental animals were found to prevent chronic infections but the immunity induced by them depends on induction of an antibody response, which was extremely short lived (Houghton et al., 1997). Puig et al., (2004) found that E1E2 protein immunization induced a delay in virus replication but did not prevent chronic infection.

In this respect, the current study presents a preclinical analysis of different formulations for a core vaccine against HCV, using the C57Bl/6J mouse model. Given the complexity of IFN-γ regulation, it is not surprising that inbred mouse strains vary in their ability to secrete this cytokine; for example T lymphocyte of C57BL/6 and C3H mice secrete significantly higher amount of IFN-γ compared with T lymphocyte of BALB/c and B10D2 mice. Increased IFN-γ production in these strains is associated with greater resistance to bacteria and viruses (Autenrirth 1994).

Based on this, we hypothesized that a vaccine should aim at reactivating T cell responsiveness (Hu 2005). Seder et al., (2008) and Shoukry et al., (2004) stated that, a significant cell-mediated immune response is important for protection against HCV core antigen.

Technology for liposome formation described in our work is characterized by high-yield vaccine entrapment in vesicles of an average size that ranges from about 14.9 to 103 nm, under conditions that preserve the biological activity of such labile antigen. Liposomes have successfully been used as drug carriers and have also been proposed to be carriers of Ags and adjuvants to induce immune responses (Peek et al., 2008; Fenske et al., 2008). However, data suggested that most, if not all, adjuvants enhance T and B cell responses by engaging components of the innate immune system, rather than by direct effects on the lymphocytes themselves (Pulendran et al., 2010). In vivo use of liposomes has been made by every conceivable route, including the intravenous, intramuscular, subcutaneous, intrathecal, intratracheal, oral, intranasal, and topical routes. Liposomes, unlike other adjuvants do not induce granulomas at the site of injection or hypersensitivity reactions in pre-immunized animals when the antigen is given in the entrapped form (Nasir et al., 2008). This is why; liposomal delivery has long been preferred due to its safety, comparative ease of assembly, as well as the diverse range of morphologies, sizes, tissue targeting and controlled release characteristics (Jiao et al., 2004).

We designed different formulations for HCV core Ag vaccine. The first formulation
encompassed core Ag encapsulated in liposomes. The significantly very high rise in proliferation is braced by the observation of Chen et al., (2006) who showed that fusion antigens could stimulate mice to produce high-level CTL response. This also agrees with Murata et al., (2003) who found that immunization with liposomal peptide vaccines confer partial protection from infection with recombinant vaccinia virus expressing HCV sequences. It is further supported by the conclusions of Ludewig et al., (2000) who found complete protection in immunised C57/BL6 mice against choriomeningitis virus after using liposomal formulations. Moreover, liposome-entrapped plasmid DNA encoding the hepatitis B surface antigen (HBsAg) circumvented the difficulties facing naked DNA immunization against hepatitis B. It facilitated the uptake of DNA by antigen presenting cells (APC) and protected DNA from nuclease attack (Look et al., 2010).

It is clear that HCV-specific CD4\(^+\) T cells also play a critical role and are central to the initiation and maintenance of adaptive immunity. In fact, viral clearance has been associated with CD4\(^+\) T cell activation (Freeman et al., 2003). Th1 response has long been accompanied with IFN-\(\gamma\) secretion, leading to activation of macrophages, CTL, and a high level of IgG2a antibodies in mice. Th1-type response is highly desirable for vaccines targeting chronic viral diseases or infections caused by intracellular pathogens. Analyses of the cytokine profiles of HCV-specific T cells showed that persons with a Th1 profile (antigen-dependent production of IL-2 and IFN-\(\gamma\)) are more likely to experience viral clearance (Ming et al., 2003). Bertoletti et al., (1997) revealed that the majority of liver infiltrating T cells in chronic hepatitis were Th1 cells that secrete IFN-\(\gamma\). This may be supported by the finding of Bertoletti et al., (2000) and Thimme et al., (2001) who demonstrated the importance of the IFN-\(\gamma\) production as a strategy to inhibit viral replication in absence of cytotoxic T cell-related liver pathology. In this respect, Himoudi et al., (2002) suggested the identification of epitopes inducing substantial IFN-\(\gamma\) secretion with little importance to cytotoxicity to influence the vaccine development.

In this context, liposomes encapsulating Ag in our study showed also the highest rise in IFN-\(\gamma\) secretion and this coincides with our findings of increased T cell response. It is of great value as a protective agent as the possible role of IFN-\(\gamma\) in clearing up the infection has been put worth. This is further reinforced by the observation that IFN-\(\gamma\) was detected from liver of the chimpanzees which effectively suppressed the infection and coincided with increases in HCV-specific T cell responses (Zubcova et al., 2009). IFN-\(\gamma\) level in our study increased significantly after stimulation and this agreed with Zhang et al., (2010) who found that after injection of con A, the blood level of IFN-\(\gamma\) increased dramatically. In addition, Faggioni et al., (2000) revealed that, IFN-\(\gamma\) mediate con A induced hepatitis.

Other studies considered coupling of HCV peptides to the surface of liposomes without encapsulation (surface-linked liposomal peptide). This had been served as efficient vaccine vehicles for the induction of immunity mediated by CD8\(^+\) T cells. Takagi et al., (2009) exhibited an efficient antiviral response in immunized mice with liposomal peptide. They observed an induced Ag-specific memory CD8\(^+\) T cells in the absence of CD4\(^+\) T-cell help, which could be shown in CD4-knockout mice. Taneichi et al., (2010) supported this finding and suggested that surface-coupled liposomal antigens could be presented by APCs to CD8\(^+\) T cells via MHC class I molecules. This antigen preparation was expected to be applicable for the
development of viral CTL-based peptide vaccines that effectively eliminate virus-infected cells especially for those viruses that evade humoral immunity by varying their surface proteins, such as HCV. The mechanisms underlying the induction of different immunogenicities of the peptides by coupling to the surface of liposomes are associated with the differences in the antigen presentation mechanisms.

Alternatively, and in concordance with our findings, Gouri et al., (2012) observed a dual up regulation in Th1 and Th2 genes and serum antibody isotype upon liposome encapsulation of viral peptide indicating a balanced immune response and its enhancement. Chandra et al., (2012) encapsulated siRNAs targeted to the highly conserved 5'-untranslated region of the HCV genome into lipid nanosomes and showed that siRNA can be repeatedly delivered to 100% of cells in culture using nanosomes without liver injury or histological toxicity and dramatically reduced HCV replication in both the replicon and infectious cell culture model. In agreement with our study Li et al., (2001) discovered that liposomes co-encapsulating CpG ODN with the selected antigen could enhance the immune responses against T-independent antigen due to protection from extracellular degradation and neutralization by circulating antibodies.

Empty liposomes group of mice showed an elevated cellular proliferation than the saline injected group, however, this elevation was significantly lower than liposomes with core Ag group. This could be explained by the findings of Norbert et al., (2001) who found that liposomes with or without additional antigens, can be used as carriers of immunomodulators including macrophage activators such as muramyl peptides and cytokines. Also Takagi et al., (2009) exhibited a complete protection and highly efficient antiviral responses in immunized mice even when the Ag dose was reduced to as low as possible in liposomal peptide. The significant lower proliferation and IFN-γ production in this group compared to liposomes with core Ag group may be explained by Dahari et al., (2010) study that found a significant protective immune responses in chimpanzees with the inclusion of structural HCV proteins in the vaccine.

Core Ag injected group showing a lower proliferation may be supported by the failure of DNA vaccines containing HCV core protein to induce a successful cellular and humoral response in mice models but with more encouraging results in primate studies (Polakos et al., 2001).

The slight elevation of IFN-γ in this group may be explained by the fact that core vaccines employing the core protein have demonstrated specific CD4+ T-cell response in some experiments (Isaguliants et al., 2004). Using liposomes with the Ag was highly significant than the Ag alone and this also agreed with Large et al., (1999) who found that vaccinia viruses, recombinant for HCV core protein, failed to elicit cellular immune response in mice. They suggested that, the HCV core protein was responsible for the failure in eliciting immune response by suppressing the host response. Interestingly, Engler et al., (2004) found that in mice immunized with liposomes containing CpG, the IFN-γ secreting peptide-specific T cells were expanded. Obst et al., (2007) suggested that, subunit vaccine antigens are generally small inert peptides, their administration without any carrier molecule leads to their rapid removal by the host and therefore little, if any, immune response is mounted. In order to increase the duration of antigen presentation to APCs and subsequently T cells for the initiation of memory responses, liposomes can be used to simultaneously delivery antigen. T-cell vaccines that elicit effective immune responses against HCV in
chimpanzees may create greater immune pressure for viral mutation. This data and mouse immunization studies confirm this as a more efficient delivery system than direct intramuscular inoculations with naked DNA.

Jiao et al., (2003) studies showed that immunization with cationic liposomes encapsulating rNS3 and CpG induced a much higher titer of anti-HCV NS3 IgG with significantly higher levels of Th1 cytokines and lower levels of Th2 cytokine than in the mice immunized with naked rNS3. This suggested that liposome encapsulation could delay the release of the entrapped contents, thus prolonging the time of stimulation and be a good candidate vaccine to induce strong Th1 immune responses against hepatitis C viruses. Mosmann et al., (1986) and Natalie et al., (2011) also vaccinated Mice with PHB beads produced by L. lactis which displayed HCV core antigens and demonstrated an antigen specific Th1 immunity pattern shown by production of IFNAγ as well as ILA12A17. This finding was in agreement with Engler et al., (2004) who found a persistent increase in the frequency of specific IFN-γ secreting cells stimulated by liposomal formulations exclusively containing the CTL epitope or combinations with the T helper epitope HBV core respectively. Interestingly, they repeatedly found that in mice immunized with liposomes containing CpG the IFN-γ secreting peptide-specific T cells seemed to have further expanded.

In (CFA with Ag) group, we witnessed a significant rise in splenocytes proliferation, with a high stimulation index, indicating the importance of adjuvants in boosting the immune response. These results could be explained by the fact that, CFA achieves qualitative alteration of the immune response and exerts its adjuvanticity by prolonging retention and increasing the effective size of the immunogen and so promoting phagocytosis and presentation by macrophages. They stimulate the influx of the macrophages to injection site, promote local cytokines productions and modulate the immune response by preferentially stimulating Th1 (O’ Hagan et al., 2009; McKee et al., 2010). Nevertheless, this rise in cellular proliferation was significantly lower than group liposomes with the Ag.

The significant increase in IFN-γ levels in (CFA with Ag) group is supported by these findings, Qiao et al., (2003) showed that, addition of adjuvants enhance the immunogenicity of HCV-Like particles. Linblad et al., (2000) revealed that, the mycobacterial components in CFA tend to produce stronger delayed-type hypersensitivity and skew the response toward a Th1 profile. This result was also supported by Alfons et al., (2001), where TNF-a, IL-12, IL-6 and IFN-γ. Were induced in the early phrases following exposure to CFA. Stasiuk et al., (1996) showed high expression of cytokine genes in lymph nodes and spleens of mice immunized with collagen-II in CFA and Natalie et al., (2011), observed a significant increase in IFN-γ levels after vaccination with recombinant HCV core antigen in either Emulsigen or CFA. However, this high level is also significantly lower than what we had observed in liposomes with the Ag group. So, in order to achieve optimal effects, it is necessary to use a combination of adjuvants, considering the complex series of events leading to an antigen-specific activation of the immune system. In conclusion, we found that, the encapsulation of the peptides allowed the stimulation of a T cell effective response and proliferation as well as higher secretion of IFN-γ that could be further promoted by an adjuvant.

References


