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ORIGINAL ARTICLES

The Interplay of IL-2 and IFN-γ in Response to HCV Peptides in Healthy High Risk Egyptian Health Care Workers: A Pilot Study

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ABSTRACT

Characterization of virus-specific immune responses in seronegative persons, the highly risk groups such as health care workers (HCWs), may provide important clues as to the nature of protective immunity in HCV. We studied the Interleukin-2 (IL-2) and interferon gamma (IFN-γ) to different HCV peptides among 60 HCWs with and without evidence of HCV infection. Participants were classified according to the proliferation index in a CFSE proliferation assay. Results showed positive HCWs produced a higher IL-2 in response to all peptides except NS4 and a higher IFN-γ response to NS3 and 4 compared to negative HCWs. On the other hand, there was no difference in the IL-2 response with a higher IFN-γ response to NS4 and 5 in chronic HCV-HCWs. This differential pattern of IFN-γ and IL-2 production demonstrates that the magnitude and type of cytokines produced in HCV infection are critical in determining the outcome of infection.

Key words: hepatitis C, health care workers, HCV peptides, cytokines, CFSE and proliferation assay.

Introduction

Prospective studies of healthcare workers after occupational exposure have identified the average HCV transmission rate associated with contaminated needle sticks to be 1.8% (Alter, 2002). A systematic review of 22 studies reporting a total of 6956 injuries involving HCV-contaminated needles demonstrated seroconversion rates ranging between 0% and 10.3%, with a mean of 0.75%,with worldwide differences in HCV seroconversion rates. This suggests that genetic factors might provide some level of natural resistance against HCV (Kubitschke et al., 2007). Little is known about the prevalence of hepatitis C virus (HCV) among healthcare workers (HCWs) in Egypt, where the highest worldwide prevalence of HCV exists. In recent studies the prevalence of HCV antibody in Egyptian healthcare workers was found to range from 11.2% to 16.6% (Rafik et al., 2009; Abdelwahab et al., 2012) and HCV viraemia was found to be 8%, but the prevalence was not significantly different than that found among non-medical personnel. Both studies concluded that the infections were more likely to be community-acquired and not occupationally related but Rafik et. al (Rafik et al., 2009) added that workers in the dialysis unit and laboratory technicians showed significantly higher prevalence than other job groups in the same departments.

HCV-specific T-cell responses have been described in studies of exposed HCV-seronegative individuals (Kubitschke et al., 2007; Rafik et al., 2009; Abdelwahab et al., 2012; Zeremski et al., 2009) where these responses were found to be broad and associated with risk factors for HCV exposure, suggesting that they reflected true exposure to HCV in seronegative persons. Evidence for the presence of a cellular immune response was detected by a CFSE assay where a positive proliferative response to different HCV peptides was detected in 27/60 aviremic seronegative Egyptian laboratory and blood bank HCWs. (Abdelhady, 2009). Characterization of virus-specific immune responses in exposed but seronegative persons may provide important clues as to the nature of protective immunity in HCV and provide an important insight for the design of a prophylactic/therapeutic vaccine-priming Ag-specific cytotoxic T lymphocyte (CTL) response.

Our aim in this study was to research one aspect of the protective HCV immune response, the cytokine response via interleukin 2 (IL-2) and interferon gamma (INF-γ), to different HCV peptides in a high risk population of Egyptian HCWs to elucidate the effects of different HCV peptides on cytokine synthesis in a proliferative response.

Material and methods

The study included 60 HCWs (38 males and 22 females) with a mean age of 36± 10 years who worked at Ain Shams University laboratory and blood bank and were thus considered a high risk health care population.
Only 10 of them were found to be viremic (HCV PCR +ve) and were HCV antibody +ve while all the other were seronegative for HCV antibody and aviremic for HCV. Cell culture supernatants from peripheral blood mononuclear cells (PBMCs) stimulated by HCV peptides in a CSFE proliferation assay (Lyons and Parish, 1994) were obtained from available participants (47 HCWs) and stored at -80°C until assayed. The personnel included in this study were divided into two groups:

1) HCV double negative (HCWs): (negative PCR & antibody) (Group I):

This group included 37 HCWs who had normal serum AST, ALT, negative HCV antibody & were negative for HCV RT-PCR.

2) Chronic HCV (HCWs) (positive Control group): (Double Positive PCR & antibody for HCV)(Group II):

According to the proliferation index in the CFSE proliferation assay for the HCV peptides, group 1 HCWs were further classified into 2 subgroups.

Gp1a) Positive proliferation index (+ve P.I) HCWs:

Subjects whose cells showed a P.I more than the cut off values of the PI for the HCV specific peptides of the chronic group (group 2) (27 samples were available).

Gp1(b) Negative proliferation index (-ve P.I) HCWs:

Subjects in group I whose cells showed a P.I less than the cut off values of the PI of HCV specific peptides of the chronic group (10 samples).

HCV proteins used were derived from the HCV-1 sequence and encoded core (C25-aa 2-120), NS3 (C33 aa 1192-1457), NS4 (C100.3aa 1569-1931), and NS5 (NS5 aa 2054-2995).

IL-2 & INF-γ Analysis:

All cell culture supernatants were clarified by high-speed centrifugation and analyzed using Luminex xMAP technology for IL-2, and INF-γ kits supplied by R&D diagnostics which is designed for use with the Luminex100™, Analyzer manufactured by Luminex Corporation. (R&D systems, Minneapolis, United States of America). All protocols for this study were reviewed and approved by the Ain Shams Faculty of Medicine ethics committee and all participants provided written informed consent.

Statistical analysis:

IBM statistical package for social sciences (SPSS statistics) (V. 19.0, IBM Corp., USA, 2010) was used for data analysis. Data was expressed as Median and Percentiles for quantitative non-parametric measures utilizing Wilcoxon Rank Sum test, Ranked Spearman correlation test and Kruskall Wallis test.

Results:

IL-2 and IFN-γ response to HCV peptides produce a similar cytokine pattern within each individual group:

All of the HCV peptides (Core, NS3, NS4,and NS5) studied produced a similar cytokine response (IL-2 and INF-γ) with no significant difference between them in each group i.e. there was no predominant peptide (Table 1). Correlations between IL-2 and INF-γ in response to the different HCV peptides within each group were done and Spearman correlation coefficient (r_s) is reported (Table2).

The core peptide produced a significant positive correlation between IL-2 and INF-γ only in the positive HCWs. Whereas NS4 produced a significant positive correlation only in negative HCWs, NS5 produced such a correlation in both positive and negative HCWs. The only significant correlation detected between IL-2 and INF-γ in chronic group was for NS3 which showed a negative correlation between both cytokines while NS3 produced a positive correlation in the positive HCWs. Human interleukin 2 promotes expansion and induces peripheral T lymphocytes to produce immune IFN-γ and does not in contrast, induce non-T cells and macrophages to produce IFN-γ (Kasahara et al., 1983; Helen et al.,1998; Kasahara et al.,1983).

In the positive HCWs the observed correlation between IL-2 and IFNγ induced by core,NS3 and NS5 is probably due to IL 2 induced IFN-γ production from CD8+ T cells and these results typically display a type 1 cytokine profile (IFN-γ derived from in vitro Ag-stimulated proliferating memory T cells).
Differentiating characteristics of the cytokine response between groups:

There was no difference in IL-2 response to all the HCV peptides between positive HCWs and the chronic HCV HCWs group. However, when the IL-2 response was compared between the positive HCWs and the negative HCWs all peptides except NS4 resulted in a significant higher response. As for the chronic group only the core peptide resulted in a higher IL-2 response among chronic HCV HCWs (gp2) when compared to the negative HCWs. The prevailing paradigm of T lymphocyte control of viral replication is that the protective capacity of virus-specific CD8+ T cells is directly proportional to the number of functions they can perform, with IL-2 production capacity being critical (Makedonas et al., 2010).

Regarding the IFN-γ response only NS4 and NS5 peptides resulted in a significantly stronger IFN-γ response in the positive HCWs (gr1a) compared to chronic HCV HCWs (gp2) and a higher response to NS3 and NS4 peptides when compared to the healthy non proliferative group (gp1b) (Brady et al., 2003). Peripheral blood mononuclear cells (PBMC) from chronically HCV-infected patients secrete IL-10, but not IFN-γ, in response to HCV nonstructural protein 4 (NS4) (Brady et al., 2003). In our population, only NS3 peptide evoked the strongest INF-γ response but this was not significantly different in chronic HCV HCWs and positive HCWs but a positive correlation between IL-2 and IFN was found in the positive HCWs and a negative correlation was found in the chronic HCV HCWs. The INF-γ response to the Core peptide didn’t show any difference between all groups.

Recent strategies of assessing human antiviral T cell responses focus on the quality of the T cell response, the more effector functions that constitute the overall response, and the more protective the response is. The functions quantified simultaneously include up regulation of interferon gamma (IFN-γ) and interleukin-2 (IL-2) (Harari et al., 2006; Pantaleo and Harari, 2006; Zimmerli et al., 2005). The cytokine response in positive healthcare workers is a more protective response at least in relation to NS4 and NS5 whereas the chronic HCV HCWs do not show this type of response to any of the peptides tested.

The INF gamma response to NS4 in positive HCWs is significantly higher than in negative HCWs whereas there was no significant difference in the IL-2 response to this peptide. There was no correlation between both cytokines in response to NS4 in positive HCWs and there was such a correlation in the negative HCWs. This indicates another source for INF than that induced in response to IL-2 in the positive HCWs which may be due to an innate immune response. As for NS3 peptide the IL-2 response in the positive HCWs was a higher than that in the negative HCWs with the presence of a positive correlation with IFN which could not be detected in the negative HCWs. The HCV NS3 peptide seems to stimulate a predominantly independent IFN-γ production in naïve individuals. IL-2 plays a critical role in the regulation of Th1 and Th2 responses and impacts both IL-12-dependent and -independent IFN-γ production (Chang et al., 2002).

HCV viral load correlates with IL-10 production, while low HCV RNA level associates with high IL-2 and IFN-γ production. The differential patterns of IFN-γ and IL-2 production between clearers and persisters demonstrates that the magnitude and type of cytokines produced early in HCV infection may be critical in determining the outcome of infection (Flynn et al., 2011).

Table 1: IL-2 and IFN-γ response to different HCV peptides in the Studied Groups (Kruskall Wallis).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cytokines</th>
<th>Peptide</th>
<th>Core</th>
<th>NS3</th>
<th>NS4</th>
<th>NS5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Positive HCWs</strong></td>
<td>IL-2</td>
<td>16.855</td>
<td>36.18</td>
<td>17.405</td>
<td>20.18</td>
<td>0.209</td>
</tr>
<tr>
<td></td>
<td>IFN-γ</td>
<td>2.16</td>
<td>11.4</td>
<td>3.31</td>
<td>2.0</td>
<td>0.662</td>
</tr>
<tr>
<td><strong>Negative HCWs</strong></td>
<td>IL-2</td>
<td>1.6</td>
<td>6.8</td>
<td>3.7</td>
<td>3.33</td>
<td>0.196</td>
</tr>
<tr>
<td></td>
<td>IFN-γ</td>
<td>0.83</td>
<td>1.36</td>
<td>0</td>
<td>0.83</td>
<td>0.588</td>
</tr>
<tr>
<td><strong>Chronic HCWs</strong></td>
<td>IL-2</td>
<td>10.9</td>
<td>16.1</td>
<td>8.7</td>
<td>28.33</td>
<td>0.807</td>
</tr>
<tr>
<td></td>
<td>IFN-γ</td>
<td>0.065</td>
<td>0.35</td>
<td>0.28</td>
<td>0.175</td>
<td>0.383</td>
</tr>
</tbody>
</table>

Table 2: Correlation Between IL-2 and IFN-γ Within Individual Groups.

<table>
<thead>
<tr>
<th>Variables</th>
<th>INF-γ&amp; IL-2</th>
<th>P</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive HCWs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Core</td>
<td>0.514</td>
<td>0.014</td>
<td>S</td>
</tr>
<tr>
<td>NS3</td>
<td>0.51</td>
<td>0.024</td>
<td>S</td>
</tr>
<tr>
<td>NS4</td>
<td>0.226</td>
<td>0.311</td>
<td>NS</td>
</tr>
<tr>
<td>NS5</td>
<td>0.512</td>
<td>0.015</td>
<td>S</td>
</tr>
<tr>
<td>Chronic HCWs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Core</td>
<td>-0.395</td>
<td>0.439</td>
<td>NS</td>
</tr>
<tr>
<td>NS3</td>
<td>-0.894</td>
<td>0.041</td>
<td>S</td>
</tr>
<tr>
<td>NS4</td>
<td>0.031</td>
<td>0.935</td>
<td>NS</td>
</tr>
<tr>
<td>NS5</td>
<td>-0.213</td>
<td>0.686</td>
<td>NS</td>
</tr>
<tr>
<td>Negative HCWs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Core</td>
<td>0.403</td>
<td>0.282</td>
<td>NS</td>
</tr>
<tr>
<td>NS3</td>
<td>0.451</td>
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<td>NS</td>
</tr>
<tr>
<td>NS4</td>
<td>0.294</td>
<td>0.003</td>
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</tr>
<tr>
<td>NS5</td>
<td>0.72</td>
<td>0.029</td>
<td>S</td>
</tr>
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</table>
Discussion:

Clearance of HCV-RNA may not necessarily correlate with the appearance of acquired immunity and may be an explanation for the rather low seroconversion rate after occupational exposure to HCV (Meyer et al., 2007). Other studies indicate that selected high-risk subjects can mount an effective immune response against HCV infection in the absence of antibody production and that they may have a higher likelihood of viral clearance in that setting (Post et al., 2004).

Furthermore, the development of cellular immune responses to HCV in some seronegative healthy individuals, who were either sexual partners of HCV-infected patients or healthcare workers with possible occupational exposure to the virus, has been reported (Koziel et al., 1997; Bronowicki et al., 1997). Research in Egypt has documented CMI-positive immune responses in HCV seronegative sexual partners of patients with acute HCV Hepatitis (Kamal et al., 2004) and in household contacts of HCV patients (Al-Sherbiny et al., 2005).

Conflicting results have been reported about the magnitude, breadth, quality and duration of HCV specific immune responses in different clinical settings of HCV infection and given the difficulty in identifying persons who successfully clear acute HCV examination of immune responses in large numbers of exposed but uninfected persons provides an alternate strategy for identification of protective immune responses in HCV. Thus our aim was to examine healthy high risk health care workers who were seronegative and aviraemic to immune responses in different clinical settings of HCV infection and given the difficulty in identifying persons who successfully clear acute HCV and in household contacts of HCV patients (Al-Sherbiny et al., 2005).

The hypothesis was that the type and level of key cytokines produced during their HCV exposure may be a critical determinant of viral clearance.

CD8+ T cells recognize and respond to only a very small fraction of potential viral epitopes although the viral pathogen may encode thousands of potentially immunogenic determinants. One of the factors that contribute to CD8+ T cell immunodominance is the number of naïve T cells that express complementary T cell receptors (Bronowicki et al., 1997). The immunodominance hierarchies within HLA-A2-restricted HCV-specific CD8+ T cell responses was recently studied to determine if they are linked to naïve-precursor frequency (Schmidt et al., 2011). Precursor frequencies to Core 132, NS3 1406 and NS5B 2594 specific epitopes were conserved across the healthy donors although they varied significantly between epitopes suggesting that a clear hierarchy of naïve HCV-specific CD8+ T cell precursor frequencies exists in healthy donors (Schmidt et al., 2011). The NS3 1406 epitope had the highest naïve-precursor frequency in healthy donors and the most frequently targeted epitope in chronically HCV-infected patients, both in vivo and after in vitro stimulation whereas the Core 132 epitope was rarely targeted in most studies. (Lechner et al., 2000; Spangenberg et al., 2005; Chang et al., 2001; Lauer et al., 2004). In our HCVs of all the HCV peptides studied NS3 peptide (1192 to 1457) evoked the highest IL-2 response in both positive and negative HCWs whereas the core peptide produced the lowest IL-2 response. The difference in the IL-2 induced was significantly higher in positive HCWs than the negative HCWs for all peptides except NS4. The presence of effector T cells in the positive HCWs is directed against core, NS3 and NS5.

As for the chronic cases the most predominant IL-2 response was not obtained by NS3 peptide but by NS5 and the lowest IL-2 response was not by the core but by the NS4 peptide. The only significantly higher IL-2 response in the chronic group as compared to the negative HCWs was that obtained by the core peptides. When compared to the positive HCWs all the peptides produced lower level of IL-2 but with no significant difference.

This pattern of IL-2 production was further differentiated by the pattern of IFN-γ production where we found no significant difference between the negative HCV and the chronic cases to all peptides tested. However the positive HCWs showed a significantly higher IFN-γ in response to NS4 and NS5 than the chronic cases and a significantly higher IFN-γ in response to NS3 and NS4 than the negative HCWs. This IL-2-INF-γ relation was

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<tr>
<td>NS4 2594</td>
<td>0</td>
<td>0.004</td>
<td>NS4 2594</td>
<td>0</td>
</tr>
<tr>
<td>NS5 2867</td>
<td>0.28</td>
<td>0.245</td>
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Table 3: Comparison between the IL-2 responses to different HCV peptides within the studied groups.

Table 4: Comparison between the INF-γ responses to different HCV peptides within the studied groups.

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further confirmed by the positive correlation detected between them in positive HCWs for all the peptides except NS4 and by the positive correlation for both cytokines induced by NS3 and NS4 in negative HCWs.

The differential patterns of IFN-γ and IL-2 production between our positive HCWs (clearers) and the chronic cases (persisters) demonstrate that the magnitude and type of cytokines produced in HCV infection are likely to be critical in determining the outcome of infection. IL-2 cytokine secretion in response to a panel of recombinant HCV antigens in patients with acute HCV has been studied previously and showed that patients with self-limited disease had a significant secretion of IL-2 (Semmo and Klenerman, 2007) and those who developed chronicity had significantly lower IL-2 (Pawlowska et al., 2005) and IFN-γ production than those cases who recovered (Klenerman et al., 2002; Williams and Bevan, 2007). The emergence of an IFN-γ−, CD58+ non-cytopathic CD8+ T-cell response might be key to the host’s victory over HCV (Bell, 2002).

During persistent viral infections, specific T cells can become functionally inert, incapable of cytotoxicity and of producing IL-2 or IFN-γ (Gruener et al., 2004) described as the ‘stunned’ phenotype in HCV infection. The IL-2 response to the different peptides tested in our study did not show any differences between the positive HCWs and chronic cases which does not support the stunned phenotype for IL-2. However this was not true for IFN-γ where a stunned phenotype was evident for almost all of the nonstructural peptides (NS4 and NS5 with near significance for NS3 p = 0.055). The higher amount of IFN-γ produced by T-cell derived from patients who recovered from HCV hepatitis than that produced by T cells derived from patients with chronic evolution of infection has been documented (Lamonaca et al., 1999; Klenerman et al., 2002; Wedemeyer et al., 2002; Freeman et al., 2001). The majority of HCV-specific IFN-γ production was from clearers by both CD8+ T cells and CD56+ cells, with little to no production from persisters (chronic) and it was also detected more frequently when PBMC were stimulated with the HCV antigen NS4/5 (Freeman et al., 2001), CD4+ T-cell responses predominantly targeting nonstructural proteins were also associated with resolved HCV infection (Ruys et al., 2008). The lowest magnitude of responses in persons with resolved infection was to the relatively small core protein, and these responses were not significantly different from persons with chronic infection (Schulze et al., 2005). The same pattern of reactivity to non structural proteins in seronegative aviremic individuals was also recorded. Sexual partners had a higher production of IFN-γ and IL-4 by CD4+ cells against NS3 and NS5b, and CD8+ cells against NS3 and NS4b as compared with healthy controls (Yewdell, 2006).

The observed pattern of a vigorous CTL response and relatively weaker humoral response predicting disease resolution suggests that HCV persistence also relates to the pattern of cytokine release (Hashem et al., 2005). Persistence may relate to a relative insensitivity of HCV to antiviral cytokines such as TNF-α and IFN-γ (Koziel et al., 1997; Chang et al., 2001), since viral clearance was associated with a greater magnitude and broader specificity of IFN-γ and IL-2 responses (Flynn et al., 2011). Finally the frequent and low levels of HCV viremia may have been controlled by innate immune responses without a strong adaptive immunity (Meyer et al., 2007).

Cell-mediated immune (CMI) responses to hepatitis C virus (HCV) antigens in adults without seroconversion or viremia are biomarkers for prior transient infection (Hashem et al., 2005). It is stipulated that these CMI-positive HCV seronegative subjects may have had a transient very mild infection, possibly associated with low-dose exposure to the virus, which was cleared. Memory virus-specific T lymphocytes are markers of this prior exposure to HCV and this is probably reflected in the strong cell-mediated immune responses in the absence of any serologic or virologic evidence of infection in occupational or household contact with persistently infected individuals. Conventional mechanisms for sustaining T cell memory are likely to be involved, including cytokine-driven homeostatic proliferation (Welsh and Selin, 2002). A vigorous HCV-specific type 1 helper T cell CD4+ response, particularly against nonstructural proteins, is associated with viral clearance (Aberle et al., 2006). Cellular immune response may remain after the loss of anti-HCV antibodies, or persist in people who never generate a significant antibody response. It is possible that cellular immunity persists due to retained antigen, or even plausibly low-level viral replication in the liver of clinically recovered patients (Takaki et al., 2005).

Our study showed that healthcare workers remain seronegative despite repeated exposure to the virus through various types of contact with HCV patients’ biological fluids. This suggests that there may be large numbers of persons who are usually repetitively exposed and who may possibly develop immunity and that clearance of low levels of HCV viremia is possible in the absence of a strong adaptive immune response which might explain the low seroconversion rate after occupational exposure to HCV. It is very likely that the true rate of individuals in the general population who had contact with HCV is underestimated, in particular in high risk persons such as iv-drug addicts and in medical health professionals.

This may indicate a hidden reality in Egypt where high risk healthcare workers could be a potential source of infection due to occult hepatitis with low-level viral replication in the liver leading to a reinfection and thus maybe one of the factors leading to maintenance of the high prevalence of HCV in Egypt. An increased understanding of the cytokine environment early in HCV infection is important for the development of novel therapeutics to facilitate viral clearance.
References


