Serum cytokine profile in hepatitis C virus carriers presenting cryoglobulinaemia and non-organ-specific autoantibodies

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1. Introduction

The chronic infection caused by HCV provokes important B cell dysfunction, which is mainly represented by cryoglobulinaemia and non-organ specific autoantibody (NOSA) production [1–3]. Such autoimmune reactivity has been clinically associated with glomerulonephritis, peripheral neuropathy, and secondary Sjögren Syndrome and B lymphoma [4–6]. In recent reports, hepatitis C cryoglobulinaemia has been associated with increased serum IL-2, IL-4, IL-5, IL-10, IFN-γ and BAFF. In HCV carriers with cryoglobulinaemia, the levels of IL-2, IL-4, IL-5, IL-10, IFN-γ and BAFF were significantly higher than in controls [7–11]. However, the serum cytokine profile of HCV carriers presenting anti-nuclear antibody (ANA), also known as autoantibodies, is still incompletely known and its elucidation may contribute for the knowledge of the mechanisms involved in autoimmune hepatitis C and also to improve the clinical management of these subjects.

This work investigated the serum cytokine profile (IL-2, IL-4, IL-5, IL-10, IFN-γ and BAFF) of hepatitis C virus (HCV) carriers with autoimmunity. Forty-seven HCV carriers and 28 healthy controls were evaluated. Cytokine levels were measured by ELISA. Patients and controls presented similar levels of IL-2, IL-4, IL-5, IL-10, IFN-γ and BAFF. Cryoglobulinaemic HCV carriers had increased IL-2, IL-4, IL-5, IL-10, IFN-γ and BAFF. IL-2 level was decreased in HCV carriers with rheumatoid factor in comparison with those that were RF-seronegative. Patients with HCV cryoglobulinaemia had serum IL-2 levels above the upper limit of normality, IL-5 and BAFF were significantly increased when compared with those without this autoantibody. Interleukins IL-4 and IL-10 were not associated with autoimmunity.

2. Results

2.1. Demographic and laboratory features of HCV carriers

The median age was the same in both women and men infected with HCV (47.0 and 47.5 years, respectively). The infection by HCV genotype 1 was the most prevalent (31/47, 65.9%), followed by those caused by HCV genotype 3 (13/47, 27.7%) and genotype 2 (3/47, 6.4%). Cryoglobulinaemia was detected in 30 out of 47 carriers (63.8%), and it was more prevalent in HCV infected men than in women (80.0% against 45.4%, p = 0.018). The serum level of ALT was unaltered in female HCV carriers (22.5 U/L, 14.5–33.2 U/L), and it rarely was found above the reference upper limit of 35 U/L (<20%). In male HCV carriers, the median of ALT was 43 U/L (20–70 U/L; p = 0.022). Moderate elevation of ALT above the upper reference value of 45 U/L was more frequently found in men (12/25, 48.0%) and women, ranging from 53 U/L to 129 U/L (p = 0.032). The following histological score was observed in 30 HCV carriers whose liver biopsy was clinically recommended: F0–2 (9/30, 30.0%) and F3–4 (11/30, 36.7%).

The aim of this work was to investigate the profile of serum cytokines of HCV carriers that had cryoglobulins and were seropositive for non-organ specific autoantibodies.
The most prevalent autoantibodies in HCV carriers were RF-IgM (44.6%), followed by ASMA (34.0%), ANA, IgM aCL and β2GPI IgA (25.5%), RF-IgA (24.3%) and β2GPI IgM (19.1%). In contrast, autoantibodies as ANCA, IgG aCL and IgG anti-β2GPI were rarely detected (<10.0%).

No HCV genotype was associated with the presence of cryoglobulin or any type of non-organ specific autoantibody. Only antinuclear autoantibodies were found in the control group, in three healthy individuals without clinical signs of autoimmune disease.

2.2. Serum level of cytokine

No difference was observed in the serum levels of IL-2, IL-4, IL-5, IL-10 and IFN-γ when HCV and control groups were compared (p > 0.05, Table 1), nor cytokine levels were altered in the HCV with advanced fibrosis (F3–4). However, subjects carrying the HCV genotype 1 had more serum IL-2 (9.7 pg/mL, 5.9–14.3) than patients infected with HCV genotype 3 (5.9 pg/mL, 5.2–9.6, p = 0.004). The serum levels of BAFF were correlated with ALT levels (r = 0.33, p = 0.027) (Fig. 1).

Compared with non-cryoglobulinaemic, cryoglobulinaemic HCV carriers had increased serum levels of IL-2 (9.8 pg/mL, 6.4–14.2 pg/mL vs. 5.9 pg/mL, 5.1–9.6 pg/mL, p = 0.013), IL-5 (45.6 pg/mL, 36.9–70.2 pg/mL vs. 37.6 pg/mL, 34.1–37.9 pg/mL, p = 0.018) and BAFF (531.2 pg/mL, 437.7–594.8 pg/mL vs. 439.1 pg/mL, 319.7–530.1 pg/mL, p = 0.050). HCV carriers that were seropositive for IgA anti-β2GPI had increased levels of these cytokines when they were compared with HCV carriers that were seronegative for this autoantibody: IL-2 (115 pg/mL, 8.2–19.3 pg/mL vs. 6.6 pg/mL, 5.8–10.0 pg/mL, p = 0.009), IL-5 (59.0 pg/mL, 37.8–96.3 pg/mL vs. 37.3 pg/mL, 33.6–51.0 pg/mL, p = 0.018) and BAFF (579.6 pg/mL, 453.0–627.3 pg/mL vs. 424.6 pg/mL, 379.4–519.9 pg/mL, p = 0.039). Interleukin-2 was increased in the sera of HCV carriers that were seropositive for antinuclear antibodies in comparison with carriers that were ANA seronegative (10.5 pg/mL, 6.7–23.9 pg/mL against 7.6 pg/mL, 5.8–10.0 pg/mL, p = 0.044). The levels of IFN-γ were diminished in HCV carriers that were seropositive for RF-IgM (9.6 pg/mL, 7.1–15.0 pg/mL when they were compared with HCV carriers that were RF-IgM seronegative. (12.2 pg/mL, 9.5–21.0 pg/mL, p = 0.035).

Cytokine levels were similar in HCV carriers that were seronegative or seropositive for smooth muscle autoantibodies. No alteration in the serum levels of IL-4 and IL-10 was caused by autoimmunity in HCV carriers (p > 0.05).

The following cytokines were correlated in HCV carriers: IL-2 with IL-5 (r = 0.71, p < 0.0001), IL-2 and IFN-γ (r = 0.35, p = 0.015) and also IL-5 and IFN-γ (r = 0.35, p = 0.015) (Figs. 2–4).

3. Discussion

In this work, we investigated the serum cytokine profile of HCV carriers presenting extra-hepatic manifestation of autoimmunity, which was not influenced by their gender nor associated with the stage of fibrosis. However, the serum levels of BAFF were correlated with ALT levels as also observed in autoimmune hepatitis [11]. No difference was observed in the levels of these cytokines when control and HCV groups were compared. However, we verified that HCV carriers had a marginal decrease in IL-2 level, which was more pronounced in HCV carriers infected with genotype 3, suggesting an important loss of CD4+ T cells secreting IL-2 in these individuals [12]. Nevertheless, the prevalence of autoimmune biomarkers in the carriers infected with HCV genotype 1 or HCV genotype 3 was similar. Although there is some controversy about the cytokine profile of HCV carriers, which may be caused by difference in experimental conditions or cytokine source, our findings of similar serum levels of IL-2, IFN-γ, IL-4 and IL-10 in both HCV carriers and healthy controls are in accordance with those from studies performed outside Brazil [13–16].

Interleukin-2 is a cytokine produced by both Th1 and uncommitted primed precursor T cells (Thpp) that modulates tolerance mechanisms such activation-induced cell death and plays an important role in the homeostasis of regulatory T cells, whose deficiency has been associated with lymphoid hyperplasia, lethal autoimmunity and impaired Treg cell production in mice [17,18]. Here we observed an increase in the serum level of IL-2 in HCV carriers presenting cryoglobulinaemia and that were seropositive for ANA and IgA anti-β2GPI suggesting that other immune mechanisms probably not associated with IL-2 deficiency may be involved in the extra-hepatic manifestation of autoimmunity in these subjects.

We verified that cryoglobulinaemia in Brazilian HCV carriers was associated with increase in serum levels of BAFF, which is a cytokine that prolongs B lymphocyte survival and is associated with autoimmunity [19], confirming two previous reports on

![Fig. 1. Correlation between the serum levels of ALT and BAFF in HCV carriers.](image1)

![Fig. 2. Correlation between the serum levels of IL-2 and IL-5 in HCV carriers.](image2)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Healthy control n = 28</th>
<th>HCV carriers n = 47</th>
<th>p</th>
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<tr>
<td>IL-2</td>
<td>10.3 (7.1–14.3)</td>
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<tr>
<td>IL-4</td>
<td>5.2 (4.4–5.9)</td>
<td>5.5 (4.7–6.8)</td>
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<tr>
<td>IL-5</td>
<td>43.5 (35.7–56.4)</td>
<td>37.7 (35.0–67.3)</td>
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<tr>
<td>IL-10</td>
<td>25.3 (21.6–31.9)</td>
<td>25.7 (20.4–34.9)</td>
<td>0.673</td>
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<tr>
<td>IFN-γ</td>
<td>13.3 (9.5–18.0)</td>
<td>16.6 (8.8–16.6)</td>
<td>0.755</td>
</tr>
<tr>
<td>BAFF</td>
<td>424.6 (379.4–519.9)</td>
<td>496.8 (411.3–582.3)</td>
<td>0.111</td>
</tr>
</tbody>
</table>

* Median and IQR.
elevated serum BAFF in chronic hepatitis C [7–9]. In addition, we showed for the first time that increased BAFF levels may be detected in HCV carriers that are seropositive for IgA anti-β2GPI. Together, these findings suggest that BAFF plays an important role in autoimmunity in chronic hepatitis C.

Although the analysis of cytokines produced by stimulated-peripheral blood mononuclear cells (PBMC) from patients chronically infected with HCV has demonstrated an absence of predominance of Th2 immune response in these subjects [20], we verified that HCV carriers with cryoglobulinaemia and anti-β2GPI IgA antibodies had increased serum levels of IL-5, without any increase in the serum level of IL-4. Interleukin-5 is a cytokine mainly involved in the allergic inflammation and eosinophilia, which induces IgA production in synergy with transforming growth factor beta (TGF-β), a cytokine that participates of liver fibrogenesis in this viral infection [21–23]. Here IgA autoantibodies were represented by β2GPI IgA antibodies and also by rheumatoid factor of this isotype, which have been reported also to occur in about one-third of cryoglobulinaemic patients chronically infected with HCV living in other geographic region [24]. However, in addition to IgA autoantibodies, antibodies of this isotype to HCV recombinant proteins Co.120, NS3, E1.340 and HVR-1 peptide of E2 protein have been demonstrated during chronic hepatitis C, which may be also induced by IL-5 and TGF-β [25].

Although RF-IgM were detected alone or together with RF-IgA in HCV carriers, confirming a previous study no citrullinated protein IgG antibodies were found in these subjects [26]. They also had unaltered serum level of BAFF, and clinical arthritis was rarely registered in their medical files. Thus, both RF-IgM and RF-IgA may be natural polyreactive autoantibodies induced by IL-5, which represent the immune response of B-cells to HCV infection, without any link with rheumatoid arthritis or hepatitis C related arthritis [27]. However, HCV carriers that were seropositive for RF-IgM had decreased serum levels of IFN-γ as described in hepatitis C related arthritis, which may be associated with the suppressing activity of CD4+CD25+ regulatory T cells on the production of this cytokine [28].

Our finding of unaltered serum IL-10 levels in HCV carriers contrasted with those of previous studies, which reported either increase or decrease in the serum level of this cytokine in subjects chronically infected with this virus [29,30]. However, it is in accordance with the previous reports of unaltered IL-10 level in sera from patients with chronic hepatitis C or in culture supernatants of mitogen-stimulated PBMC obtained from these individuals [13–16,31,32]. Interleukin-10 is a cytokine produced by monocytes and different subsets of regulatory T and B cells that has either anti-inflammatory or immunosuppressive activity. It can costimulate B cell activation, prolonging its survival and promoting immunoglobulin switch in this lymphocyte. In addition, IL-10 can mediate autoantibody production as documented in systemic lupus erythematosus [33–36]. Although IL-10 has been described to mediate HCV dysfunction and down-regulate the immune response of CD8+ T cells against this virus, it may down-modulate liver inflammation and the progression of liver fibrosis in this infection [37]. Thus, more detailed studies are necessary to elucidate the real importance of IL-10 in the autoimmune events observed in hepatitis C.

We concluded that the serum profile of cytokines in Brazilian HCV carriers with cryoglobulinaemia and NOSA is mainly represented by increased levels of IL-2, IL-5 and BAFF, without alteration in both IL-10 and IL-4 levels.

4. Material and methods

4.1. Patients and controls

Forty-seven untreated individuals (22 women and 25 men, median age 47.0 and 47.5 years, respectively), whose chronic hepatitis C virus infection was confirmed by clinical examination and laboratory exams of third generation enzyme-linked immunoassay (ELISA) (AXSYM System; Abbott Laboratories, Chicago, IL, USA) and RNA-Polymerase Chain Reaction (AmpliCyt R⃝ HCV Detection Kit V2.0; Roche Molecular Systems Inc., Somerville, NJ, USA), were studied. HCV genotyping was performed in these subjects using the Inno-LiPA test (HCV LineProbe Assay; Innogenetics, Zwijndrecht, Belgium). No co-infection with hepatitis B virus, HTLV or HIV was diagnosed in these HCV carriers when they consented to participate of this study. The control group was formed by 28 healthy individuals that were seronegative for B and C viral hepatitis, Chagas’ disease, syphilis, HTLV-I and HIV infection. The study was previously approved by a local Ethics Committee in Human Research.

4.2. Methods

Cryoglobulinaemia was probed using both tube and gel-diffusion cryoprecipitation [38]. Commercial ELISA kits (INOVA Diagnostics, San Diego, CA, USA) were used to investigate IgG antibodies against nuclear antigens (ANA), liver and kidney microsomal type 1 antigen (LKM-1) and cyclic citrullinated peptide (CCP). The isotypes of IgA, IgG and IgM antibodies against cardiolipin (aCL) and β2 glycoprotein I (β2GPI) were also investigated by ELISA, whereas the indirect fluorescence antibody test (IFAT) was used to detect both smooth muscle (SMA) and antineutrophil cytoplasm (ANCA) antibodies with kits from the same commercial source. Anti-IgG IgM antibodies (RF-IgM) was investigated by nephelometry (Image 800, Beckman Coulter, USA), whereas the investigation of IgA anti-IgG (RF-IgA) was performed by ELISA as previously described [39]. Liver
biopsy was performed in the patients when recommended, being the histological exam carried-out with hematoxin-eosin, Picrorius red, and Peril’s stains. Liver fibrosis was staged by the META VIR score. Serum levels of ALT were determined by automated biochemical analysis.

4.3. Serum cytokine

Serum level of BAFF/BlyS was determined by enzyme-linked immunosorbsent assay of antigen capture (R&D Systems, Inc., Minneapolis, MN, USA). Interleukins IL-2, IL-4, IL-5, IL-10 and interferon-γ were determined by the same type of immunoassay, but using ELISA Ready-SET-Go! (eBioscience Inc., San Diego, CA, USA).

4.4. Statistical analysis

The continuous variables were expressed as median and interquartile range (IQR, Q1-Q3), in accordance with their distribution in D’Agostino-Pearson (“omnibus K2”). The Mann–Whitney U test was used to compare two groups. Correlation between variables was analyzed with the Spearman test, while the association between two categorical groups was evaluated by the Fisher’ exact test. The statistical software Prism 5.0 from GraphPad was used in the analysis, whose significance was set at p < 0.05.

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References