EXTENDED REPORTS

Increased serum concentrations of soluble HLA-class I antigens in hepatitis C virus related mixed cryoglobulinaemia

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Abstract

Objective—To investigate whether quantitative alterations of both β2-microglobulin (β2m) associated HLA class I heavy chains (sHLA-I) and β2m free class I heavy chains (sHLA-FHC) in sera of patients with hepatitis C virus (HCV) infection occur and whether they distinguish patients with mixed cryoglobulinaemia (MC).

Methods—83 HCV infected patients were studied and divided into three groups: (A) without cryoglobulinaemia (n=21), (B) with polyclonal MC (n=20), (C) with monoclonal MC (n=42). Serum sHLA-I and sHLA-FHC were measured by double determinant radioimmunoassay using monoclonal antibodies: TP25.99 as catching antibody, and NAMB-1 and HC-10 as revealing antibodies. Western blot identified HLA-I isoforms.

Results—The serum concentrations of sHLA-I and of sHLA-FHC in HCV infected patients versus controls were respectively 1.3 (0.5) µg/ml (mean (SD)) versus 0.8 (0.3) (p<0.001) and 13.9 (7.1) ng/ml versus 9.2 (5) (p<0.001). sHLA-I were 1.01 (0.4) µg/ml in group A, 1.04 (0.4) µg/ml in group B, and 1.47 (0.4) µg/ml in group C (p=0.001). Statistical analysis showed a significant difference versus controls for groups B (p<0.02) and C (p<0.001). sHLA-FHC were 12.8 (8.3) ng/ml in group A, 17.2 (7.1) ng/ml in group B, and 12.9 (6.2) ng/ml in group C (p<0.02). A significant difference versus controls for each group was found (p<0.02, p<0.001, and p<0.02, respectively). Different patterns of sHLA-I isoforms were observed.

Conclusions—Increased serum concentrations of sHLA-I and sHLA-FHC characterise HCV infected patients. The highest sHLA-I concentrations seem to distinguish patients with monoclonal MC. In this last condition sHLA could play a part in the HCV escape and in B cell proliferation. The significance of sHLA-FHC is still undefined.

Hepatitis C virus (HCV) infection may have extremely variable clinical consequences and is more than just a liver disease. Although the liver involvement is the most important aspect of HCV infection, other extrahepatic disorders and a plethora of immune and autoimmune perturbations have been reported.

HCV infection is a significant cause of mixed cryoglobulinaemia (MC). This condition is marked by cryoprecipitable immunocomplexes, mainly IgG-IgM rheumatoid factor (RF) in the serum. These may induce a cryoglobulinemic syndrome (CS), characterised by purpura, usually accompanied by arthralgias and weakness, and/or liver and/or kidney and/or peripheral nervous system involvement. The IgM RF may either be polyclonal (type III MC), or monoclonal (type II MC). In HCV related type II MC, a monoclonal lymphoproliferation occurs and a role of the virus in the pathogenesis of a subset of B cell non-Hodgkin’s lymphomas (NHL) has also been suggested.

Besides being expressed on most nucleated cells, β2m microglobulin (β2m) associated HLA class I heavy chains (HLA-I) are present in plasma in an immunologically functional form. High concentrations of serum HLA-class I antigens (sHLA-I) have been reported in HCV infected patients, as well as in other viral infections. In HCV infection this increase could be caused by the increased endogenous production of interferons and/or other cytokines associated with viral infection, as well as by administration of interferon for therapeutic purposes. Recently, the β2m free class I heavy chain (sHLA-FHC) has been identified in normal human serum and in systemic lupus erythematosus (SLE). It now seems that sHLA-I and sHLA-FHC participate in the immune regulation mechanism.

These findings together with the relevant part played by HCV in MC led us to investigate whether quantitative changes of both sHLA-I and sHLA-FHC in the sera of HCV infected subjects occur and whether they can distinguish patients with MC.
**Table 1 Clinical and laboratory characteristics of HCV infected patients**

<table>
<thead>
<tr>
<th>Group A without MC</th>
<th>Group B Type III MC</th>
<th>Group C Type II MC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>M/F</td>
<td>13/8</td>
<td>10/10</td>
</tr>
<tr>
<td>Age range (median)</td>
<td>23-60 (47)</td>
<td>30-66 (51)</td>
</tr>
<tr>
<td>IgM-RF Latex test</td>
<td>4 16</td>
<td>42</td>
</tr>
<tr>
<td>Low serum C4</td>
<td>0 1</td>
<td>35</td>
</tr>
<tr>
<td>Cryocrit range (median)</td>
<td>0.5-5.5 (1)</td>
<td>0.5-30 (3)</td>
</tr>
<tr>
<td>Purpura</td>
<td>0 0</td>
<td>40</td>
</tr>
<tr>
<td>Weakness</td>
<td>5 6</td>
<td>30</td>
</tr>
<tr>
<td>Arthralgias</td>
<td>1 2</td>
<td>25</td>
</tr>
<tr>
<td>Liver involvement*</td>
<td>21 20</td>
<td>34</td>
</tr>
<tr>
<td>Kidney involvement†</td>
<td>0 0</td>
<td>16</td>
</tr>
<tr>
<td>Peripheral nerve involvement‡</td>
<td>0 0 33</td>
<td></td>
</tr>
</tbody>
</table>

*Abnormal serum ALT. †Proteinuria (>0.5 g/24h) and/or haematuria (>5 RBC/HPF) and/or serum creatinine >1.4. ‡Symptomatic sensory or sensory-motor peripheral neuropathy.

**Methods**

**PATIENTS**

Eighty three unselected patients (30 men and 53 women, age range: 30–76 years; mean: 54 years) with active HCV infection detected by polymerase chain reaction at the time of diagnosis were enrolled in a cross sectional study. Forty one of 83 HCV infected patients were observed at the Infectious Disease Unit and 42 were observed at the Rheumatology Unit. A search for serum cryoglobulins was carried out and cryocrit (percentage of packed cryoglobulins) was measured by centrifugation of the serum at 4°C for 15 minutes at 1200 g, after seven days storage at 4°C. Cryoglobulins were typed by immunofixation electrophoresis. Fifty eight healthy adults (18 men and 40 women, age range: 28–67; mean: 52 years) were included in the control group. Blood samples of patients and controls were collected at 37°C and after centrifugation at 3000 rpm, sera were aliquoted and stored immediately at −40°C.

All but 11 patients receiving alpha recombinant interferon (rIFN-α2a) (Intron A, Schering-Plough Corporation) at the time of our study, had blood taken before any treatment.

**SEROLOGICAL TESTING**

Double determinant immunoassay (DDIA), a radioactive method to measure sHLA-I and sHLA-FHC, was performed in 96 round bottom well polystyrene plates (Falcon, NJ) as previously described. The following monoclonal antibodies (mAbs) were used: mAb TP25.99, recognising a determinant expressed on both β2µ associated and β2µ free HLA class I heavy chains, mAb NAMB-1, recognising the β2µ chain, and mAb HC-10, recognising a determinant preferentially expressed on β2µ free HLA-B and C heavy chains. All these mAbs were generously provided by Dr S Ferrone (New York Medical College, Valhalla, NY). Briefly, 50 µl of sera diluted 1:4 and 1:16 with 1% bovine serum albumin in phosphate buffered saline to measure sHLA-I and 1:2 and 1:8 to measure sHLA-FHC were added to wells coated with 5 µg/ml of mAb TP25.99 and incubated for 60 minutes at 37°C. Bound antigens were detected with 125I radiolabelled antibody against β2µ, mAb NAMB-1 or anti-HLA class I heavy chain mAb HC-10. All sera were tested in duplicate. Quantification (µg/ml) of the HLA-I was carried out by plotting the bound cpm mean on a reference curve included in each experiment, and obtained by serial dilutions of a known sHLA-I concentration serum sample (kind gift from Dr F Indiveri, University of Genoa, Genoa, Italy). Results of HLA-FHC were expressed as bound 125I mAb HC-10/µl.

The specificity of 125I mAb binding was checked by replacing serum samples with 1% bovine serum albumin-phosphate buffered saline and by adding serum to wells coated with the unrelated mAb R16.7. The control antiarsonate mAb R16.7 was a kind gift of Dr A Nisonoff (Brandeis University, Waltham, MA, USA). A lysate of the lymphoblastoid cell line LG-2 cells was used as a positive control.

**SDS-PAGE and western blot were performed on serum from patients and normal controls to study sHLA and sHLA-FHC isoforms.** Briefly, after preclearing with protein G-Sepharose (PGS) beads samples were incubated overnight at 4°C with NAMB-1-PGS or HC-10-PGS to detect s-HLA-I or sHLA-FHC, respectively. Bound PGS material was assessed by SDS-PAGE (12% acrylamide) in the presence of 2-mercaptoethanol. Immunoblotting was performed using a 0.45-µm nitrocellulose membrane. After protein blotting, membranes were incubated with 125I mAb TP25.99 (2 × 10^6 cpm/ml). Membranes were then autoradiographed using X-omat AR X-ray film (Eastman Kodak, Rochester, NY).

**STATISTICAL ANALYSIS**

Comparisons between groups were made by non-parametric tests (Mann-Whitney U test...
and Kruskal-Wallis test). Simple regression analysis was used to assess the significance of the correlations between the parameters measured. All p values less than 0.05 were considered statistically significant. Data are given as means (SD).

**Results**

In 62 of 83 HCV infected patients, serum cryoglobulins were found (cryocrit range: 0.5–30%). Cryoprecipitate typing showed a IgM-IgG MC in all cases. In 42 subjects a monoclonal IgMk was detected. Depending on the presence and type of serum cryoglobulins, the 83 HCV infected patients were divided into three groups: (A) patients without cryoglobulinaemia (n=21); (B) patients with type III MC (n=20); (C) patients with type II MC (n=42). Table 1 shows the clinical features and laboratory findings.

The stability of sHLA-I and sHLA-FHC concentrations was shown in preliminary experiments and less than 10% variations were found in two sequential samples of blood obtained at 48 hour intervals from five HVC infected patients and from five controls.

sHLA-I (µg/ml) and sHLA-FHC (ng/ml of bound 125I mAb HC-10) concentrations were higher in the HCV infected patients than in controls (1.3 (0.5) versus 0.8 (0.3), p<0.001; and 13.9 (7.1) versus 9.2 (5), p<0.001; respectively) (fig 1). When we compared groups A, B and C separately with controls we found significantly higher serum sHLA-I concentrations in groups B and C (1.0 (0.4), p<0.02; 1.5 (0.4), p<0.001; respectively) (fig 2A). sHLA-FHC concentrations were higher than in controls for each subgroup (12.8 (8.3), p<0.02; 17.2 (7.1), p<0.001; 12.9 (6.2), p<0.02; respectively) (fig 2B). Among the three subgroups of patients, sHLA concentrations were significantly higher in group C (p<0.001), and HLA-FHC concentrations higher in group B (p<0.02).

Eleven of the 83 HCV infected patients, three without cryoglobulinaemia and eight with type II MC (cryocrit 5%, median) were treated with rIFN-α2b at the time of our study. These 11 patients showed higher sHLA-I serum concentrations when compared with untreated patients (1.739 (0.328) versus 1.179
no differences were found in sHLA-FHC concentrations measured in 8 of 11 patients (14.75 (4.59) versus 13.82 (7.33); NS). The sHLA-I and sHLA-FHC concentrations of the IFN treated patients did not affect the differences reported in figure 2 as shown by the persistence of statistically significant differences when only the 72 untreated patients were examined.

The concentrations of sHLA-I in sera from patients with HCV infection and from controls were not significantly correlated with sHLA-FHC values (data not shown).

sHLA-I, but not sHLA-FHC, showed a significant correlation with cryocrit ($r = 0.31$; $p < 0.02$) (fig 3). No correlation was found when we considered MC II ($r = 0.22$; NS), MC III ($r = 0.21$; NS) and the eight IFN treated patients ($r = 0.10$; NS) separately.

Neither sHLA-I, nor sHLA-FHC values showed a correlation with serum aminotransferases.

Western blot analysis with $^{125}$I-mAb TP 25.99 of sHLA-I and of sHLA-FHC immunoprecipitated by anti-β₂µ mAb NAMB-1 (panel A) from sera of HCV infected patients and controls. Lanes 1 to 3: patients with MC and CS; lanes 4–5: patients with MC without CS; lanes 6–7: patients without MC; lane 8: controls; lane 9: PBS. HLA-FHC were immunoprecipitated by anti-HLA-FHC mAb HC-10 (panel B) from sera of HCV infected patients and controls. Lanes 1–2: patients with MC and CS; lane 3: patient with MC without CS; lane 4: patient without MC; lane 5: controls; lane 6: PBS. After separation by SDS-PAGE, antigens were transferred to a nitrocellulose membrane and detected by $^{125}$I-mAb TP25.99. PBS was used as a negative control.

Figure 3 Relation between serum sHLA-I concentrations and cryocrit.

![Figure 3](image-url)

Figure 4 Western blot analysis of sHLA-I and sHLA-FHC isolated from sera of HCV infected patients and controls. HLA-I were immunoprecipitated by anti-β₂µ mAb NAMB-1 (panel A) from sera of HCV infected patients and controls. Lanes 1 to 3: patients with MC and CS; lanes 4–5: patients with MC without CS; lanes 6–7: patients without MC; lane 8: controls; lane 9: PBS. HLA-FHC were immunoprecipitated by anti-HLA-FHC mAb HC-10 (panel B) from sera of HCV infected patients and controls. Lanes 1–2: patients with MC and CS; lane 3: patient with MC without CS; lane 4: patient without MC; lane 5: controls; lane 6: PBS. After separation by SDS-PAGE, antigens were transferred to a nitrocellulose membrane and detected by $^{125}$I-mAb TP25.99. PBS was used as a negative control.
Discussion
This is a comparative investigation on serum concentrations of sHLA-I and sHLA-FHC in HCV infected patients divided into three groups according to different HCV related disorder.

We found significantly higher sHLA-I and sHLA-FHC serum concentrations in HCV infected subjects than in controls. The highest sHLA-I mean values were found in cryoglobulinaemic patients (groups B and C), mainly in those with type II MC (group C). There was an overlap among patients belonging to groups with statistically different mean values of sHLA-I or sHLA-FHC; however, these results point to a trend, even if they do not identify mutually exclusive populations. Nevertheless, sHLA-I values higher than mean (2SD) of the controls were detected in six (three IFN treated) patients from group A, in four from group B and in 25 (seven IFN treated) from group C.

In healthy subjects different values of sHLA-I according to the HLA haplotype have been reported. Our patients and controls were not HLA typed and therefore, we cannot rule out that different values reflect HLA haplotype diversity. However, this is unlikely as both patients and controls belonged to the same ethnic group and high levels of sHLA-I have been described in HCV infected patients from a different ethnic group with a distribution of HLA class I allospecificities different from the population under study.

Three isoforms of HLA Class I heavy chains with molecular weight of 44, 39 and 35–37 kDa have been described to be associated with β2µ in normal serum. These three isoforms of HLA Class I heavy chain, which we also detected as HLA-FHC in sera from control donors, are not uniformly expressed in some representative sera from HCV infected patients. Analysis of the isoforms detected in HLA Class I heavy chains associated with β2µ, showed that the 39 kDa form is the most represented. In contrast, the isform distribution of HLA-FHC was similar in patients and controls.

sHLA-I increase in hepatotropic virus infections has been suggested to be caused by infected hepatocytes secretion or shedding, or release into circulation from necrotising hepatocytes with increased expression of HLA-I antigens on the cell surface membrane during viral infection. However, the relation with hepatocytolysis is still unclear. In our HCV infected patients we found no correlation of sHLA-I levels with serum aminotransferases. The same lack of correlation was also found by Hagihara et al in patients with viral hepatic disorders.

In previous reports dealing with sHLA-I serum concentrations in HCV infected patients, MC was not taken into account. We observed the highest sHLA-I values in patients with type II MC.

A different correlation between sHLA-I or sHLA-FHC serum levels and cryocrit was found. On this basis we could exclude an interference of IgM-RF and/or cryoglobulins in the assay.

It is known that the liver accounts for about 50% of circulating sHLA-I molecules in normal conditions and that HLA-I molecules are widely expressed on hepatocytes in HCV infection. However, it is conceivable that other cellular sources may account for the further increase of sHLA-I in cryoglobulinaemic patients. In fact, HCV antigens have been demonstrated in bone marrow and peripheral blood mononuclear cells of patients with MC.

Type II MC may be regarded as a low grade B cell lymphoma. In infected patients, HCV seems to drive a clonal B cell proliferation characterised by a very indolent progression because of low proliferative index and long persistence, being protected from apoptosis. Activated peripheral lymphocytes secrete high amounts of sHLA-I. Similarly, in HCV infection, B cells could actively secrete these soluble molecules, thus explaining the higher amounts observed in MC, mainly in type II MC.

Recently, Nocito et al reported increased sHLA-I serum concentrations in NHL. Our preliminary findings from four HCV infected patients with type II MC, in whom a NHL occurred (not included in the present study), confirmed high sHLA-I serum concentrations in this condition.

Because HCV circulates with VLDL, it circumvents the immune response. Experimental evidence suggests that endocytosis via the LDL receptor represents the major route of the viral entry into the cells. It has been hypothesised that in type II MC the WA cross-idiotype mRF prevents the spread of infection to other hepatocytes by blocking HCV endocytosis via the LDL receptor.

We hypothesise that sHLA-I also plays a protective part towards infected hepatocytes. The increased concentrations of sHLA-I related to HCV infection could either inhibit activity or induce apoptosis of CD8 activated cells. As a consequence an impaired T cell suppression on the B cell line may result, and a reduced cytolyis of HCV infected hepatocytes may occur.

In conclusion, significantly higher sHLA-I serum concentrations are found in HCV infected cryoglobulinaemic patients. Even if the infected hepatic cells represent an important source of these molecules, the HCV driven lymphoproliferation occurring in MC may further influence serum values of sHLA-I. We suggest that sHLA-I serum concentrations distinguish different expressions of the HCV infection, mainly type II MC. In this condition sHLA-I could reduce cytolyis of HCV infected cells. An increase in these molecules could be a mechanism to control T cell expansion in response to chronic HCV exposure. The significance of sHLA-FHC serum concentrations has still to be defined.
Soluble HLA-class I antigens in HCV related mixed cryoglobulinaemia


