The effect of Roferon A and TFX on the selected subpopulations of the peripheral blood lymphocytes in patients with chronic hepatitis C

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Abstract
Chronic hepatitis C (CHC) is a vital medical problem. The inflammatory process in the liver may lead to life threatening consequences, such as cirrhosis or hepatocellular carcinoma. Pathogenesis of CHC has not been fully recognized which influences the efficacy of currently used methods of treatment.

Aim of the studies: Evaluation of the changes in percentage values and absolute number of selected subpopulations of the peripheral blood lymphocytes in the course of the therapy in relation to the obtained results.

Material and Methods: The study was carried out on adult patients (16 females and 20 males), aged 21-63 years treated with combination of IFN-α and TFX for 24 weeks. Evaluation of absolute numbers and percentage of subpopulations of peripheral blood lymphocytes was done before and in 12th and 24th weeks of the therapy. Evaluation of lymphocyte subpopulations was performed on peripheral blood cells using standard techniques for whole blood immunofluorescence labeling. Twenty four months after the end of the therapy the patients were divided into two groups: group 1 patients with sustained viral response (SVR) (negative HCV-RNA in the serum 6 months after the end of the therapy), group 2 patients without SVR. Changes in each subpopulation of lymphocytes were compared before and during the treatment in relation to viral response. The results were analyzed statistically.

Results: Statistically significant higher percentage of lymphocytes with phenotype CD3+/DR in the investigated group as compared to the control group was found. The changes in the number of T lymphocyte subpopulation in favor of cells with cytotoxic potential were observed in group 1. Percentage and absolute numbers of CD4+ lymphocytes decreased in both investigated groups (group 1 and group 2).

Conclusions:
1. Treatment of CHC with combination of IFN-α and Thymus peptides may be a valuable supplement of therapy to currently used methods.
2. T Lymphocytes are effector cells of this therapy and during this therapy their redistribution in the organism may return to normal state.

Key words: IFN, TFX, Lymphocytes, HCV.

Introduction

Infection with virus hepatitis C (HCV) is found all over the world. It is common and tends to cause chronic hepatitis C (CHC) hence it has become one of the major health problem. So far the pathogenesis of the disease complications has not been fully recognized.

Different mechanisms caused by viruses to avoid activity of the immune system are compensated by its ability to recognize peptide sequence of virus particles. However, unusual HCV variability helps to get it out of control of the immune system. Defense reaction of the organism caused by HCV is not strong enough to fight the infection but sufficient to control viremia for years. This chronic stimulation of the immune system may be responsible for the development of pathological changes in the liver.

The development of cirrhosis may be due to survival of virus particles in hepatocytes, chronic stimulation of the immune system, induction of post inflammatory cytokine production and autoimmune reactions.

Moreover, HCV may multiply in hepatocytes, macrophages, granulocytes, lymphocytes B and also lymphocytes T [1], that is why extrahepatic virus reservoirs may be formed. Virus replication in the immune cells may influence both their activity and redistribution in the organism (through adhesive molecule expression).

The aim of the paper was to evaluate the selected subpopulations of peripheral blood lymphocytes in the course of the therapy in relation to the results of the treatment.

Material and Methods

Characteristics of the patients

The investigations were carried out on 36 adult patients (16 females and 20 males) aged 21–63 years (mean age 41.36±10.25) treated for CHC in the Clinic of Infectious Diseases, Medical Academy in Łódź, in 1997–1999. Before the investigations the following qualifying and excluding criteria for participation were used.

Patients including criteria for the study:
1. Chronic hepatitis confirmed by clinical and histopathological data before the therapy.
2. Histopathological changes at least G1S2.
3. ALT activity over 60 u/l in at least two investigations.
4. HCV antibodies in the serum.
5. HCV-RNA in the serum.

Patients excluding criteria from the study:
1. Cirrhosis of the liver.
2. Coexisting HBV infection (HBsAg in the serum).
3. Splenectomy.
4. Alcoholism or drug addiction in the interview.
5. Immunosuppressive or antiviral therapy in the last 3 years.
6. Age over 65 years.

Treatment schedule used

The patients were treated with combination of Interferon α 2a (Roferon A-Roche) and TFX preparation (Jelfa – Jelenia Gora). Roferon A, a single dose 6MU, was given subcutaneously 3 times a week (Monday-Wednesday-Friday). TFX, a single dose 10 mg, was given intramuscularly twice a week.

Forty eight week duration of therapy was planned. This time course was reduced to 24 weeks when in this time, HCV-RNA was found in the serum of the patients. Hence 19 patients underwent 48-week treatment, while 17 patients underwent 24-week treatment.

During the treatment patients were under careful medical control and underwent many laboratory tests to monitor the therapy.

For immunological investigations a reference group of 120 persons, chosen according to age, described by Zeman et al. was used [2].

Investigations

Basic laboratory tests

Basic laboratory tests of the peripheral blood: complete blood cell count, activity of the selected hepatic enzymes (ALT, AST, GGTP, ALP), bilirubin concentration, prothrombin index, were carried out in each patient before, every two weeks during the therapy and also 12 and 24 weeks after the treatment. These investigations were used to monitor the safety of the treatment.

Investigations of the viral condition

The following viral markers: HCV antibodies, HBsAg were determined once before the treatment. The investigations were done using ELISA method with Organotechnika reagents in COBAS and ROCHE apparatus.

Each patient was tested for HCV-RNA in the serum using RT-PCR method before the therapy, in 12th and 24th week of the therapy and 24 weeks after the therapy.

Patients with longer therapy (19 persons) were additionally tested in the 48th week of the therapy. Sensitivity of the HCV detection was 100 copies/ml.

Immunological investigations

Evaluation of absolute numbers and percentage of T lymphocyte subpopulation in the peripheral blood was done before the therapy and in the 12th week of the therapy. The evaluation was also done in 35 patients in the 24th week of the therapy.
Two ml of blood from the ulnar vein was obtained to a Vacutainer tube (Becton Dickinson, San Jose, USA) which contained heparin (10 U/ml). The blood was analyzed 2-4 h after collection.

Evaluation of lymphocyte subpopulation was carried out on the peripheral blood cells using standard techniques for the whole blood immunofluorescence labeling. 100 µl of the blood was shaken and incubated at room temperature with proper amount of monoclonal antibodies. Erythrocytes were eliminated by adding lysogenic fluid (Becton Dickinson) to the tube, short incubation and rinsing. Then the samples were fixed with Cellfix liquid. A set of monoclonal antibodies (Becton Dickinson) was used and the following subpopulations of lymphocytes were evaluated: CD3+ (lymphocytes T), CD19+ (lymphocytes B), CD4+ (helper lymphocytes T), CD8+ (suppressor/cytotoxic lymphocytes T), CD3+HLA DR+ (lymphocytes CD3+/DR). For evaluation flow cytometer FACS Calibur with argon laser 488 nm (Becton Dickinson) was used. SimulSET program was used for analysis. LeukoGATE reagent was used to evaluate precisely the obtained samples; this reagent due to its combination of monoclonal CD45a and CD14a antibodies allows to localize the exact position of lymphocytes in the investigated samples. The results were presented as the percentage of positive cells in the investigated sample or as absolute number (number of cells/µl) using morphological evaluation of the blood samples, which was being done simultaneously.

Statistical analyses

To assess differences in percentage values and in absolute number of selected subpopulations of the peripheral blood lymphocytes between the CHC patients and the healthy controls we used the Student’s t-test for normally distributed variables and the Mann-Whitney U-test for variables which were not distributed normally. Differences in percentage values and in absolute number of selected subpopulations of the peripheral blood lymphocytes in CHC patients before treatment and in the 3rd and 6th months of therapy were evaluated statistically by analysis of variance with post hoc comparisons. Statistical significance was defined as a value p<0.05.

Results

Sustained viral response (SVR) (negative HCV-RNA in the serum 6 months after the end of the therapy was observed in 10 patients (27.8% of all investigated patients) – group 1.

No SVR was observed in 26 patients, it is 72.2% of all investigated patients – (group 2).

The obtained results are shown in tables 1-6.

Discussion

Thymus peptides (TP) affect the maturation of T lymphocytes [3], stimulate Th lymphocytes to produce regulatory cytokines i.e. IFN-α, IFN-γ, IL-2, IL-4, GM-CSF. They increase IL-2 receptor expression on target lymphocytes. IFN-α affects cell growth, differentiation and receptor molecule expression responsible for communication between effector cells of the immune system. It has antiviral, immunomodulating, antiproliferative and also myelosuppressive activities [4]. IFN-α binds with responsive cell receptor and stimulate production of protein with antiviral activity.

Monotherapy using TP only did not prove successful as had been expected. Hence attempts to the use of TP in combination with IFN-α preparations seems to be more promising [5-9].

Many authors compare percentage of lymphocytes bearing CD3+, CD 19+, CD4+ and CD8+ antigens in the peripheral blood in patients with CHC and healthy volunteers but similar to our study no significant differences were observed [10-12]. Some authors present lower percentage of T lymphocyte subpopulations CD3+, CD4+, CD8+ [13] or only CD4+ [14] in infected patients.

Table 1. The percentage values of T lymphocytes during therapy in all investigated patients

<table>
<thead>
<tr>
<th>Subpopulation of the lymphocytes</th>
<th>Before treatment n=36</th>
<th>3 months of treatment n=36</th>
<th>6 months of treatment n=35</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+ %</td>
<td>72.56±7.48 a</td>
<td>70.64±7.23</td>
<td>68.28±8.01</td>
<td>70.3±6.7</td>
</tr>
<tr>
<td>CD19+ %</td>
<td>13.97±5.25 a</td>
<td>12.64±5.18</td>
<td>13.46±4.98</td>
<td>12.8±3.9</td>
</tr>
<tr>
<td>CD4+ %</td>
<td>47.58±6.32 a</td>
<td>44.11±6.91</td>
<td>42.75±6.65 a</td>
<td>46.8±7.4</td>
</tr>
<tr>
<td>CD8+ %</td>
<td>34.64±7.68</td>
<td>35.86±7.12</td>
<td>33.75±6.34</td>
<td>34.5±5.7</td>
</tr>
<tr>
<td>CD4+CD8+</td>
<td>1.44±0.39</td>
<td>1.30±0.43</td>
<td>1.32±0.35</td>
<td>1.4±0.35</td>
</tr>
<tr>
<td>CD3+/DR%</td>
<td>11.12±3.65 a</td>
<td>13.06±6.53 a</td>
<td>11.64±3.76 a</td>
<td>7.8±4.5</td>
</tr>
</tbody>
</table>

Mean and SD are shown.

Patients with CHC before treatment compared with control group: a – p<0.01; a – p<0.001.

Patients with CHC before treatment compared with the 3rd month of therapy: – p<0.05; – p<0.01.

Patients with CHC before treatment compared with the 6th month of therapy: – p<0.05; – p<0.01.
Table 2. The percentage values of T lymphocytes during therapy in group 1

<table>
<thead>
<tr>
<th>Subpopulation of the lymphocytes</th>
<th>Before treatment n=10</th>
<th>3 months of treatment n=10</th>
<th>6 months of treatment n=10</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3⁺/µl</td>
<td>69.40±6.16</td>
<td>68.50±4.81</td>
<td>64.50±7.43</td>
</tr>
<tr>
<td>CD19⁺/µl</td>
<td>15.5±5.62</td>
<td>13.40±6.13</td>
<td>12.80±4.94</td>
</tr>
<tr>
<td>CD4⁺/µl</td>
<td>46.10±5.38</td>
<td>42.90±7.53</td>
<td>39.60±7.53</td>
</tr>
<tr>
<td>CD8⁺/µl</td>
<td>31.60±6.40</td>
<td>36.10±5.47</td>
<td>34.70±5.40</td>
</tr>
<tr>
<td>CD4⁺:CD8⁺</td>
<td>1.50±0.30</td>
<td>1.24±0.39</td>
<td>1.17±0.25</td>
</tr>
<tr>
<td>CD3⁺/DR%</td>
<td>9.56±4.39</td>
<td>12.25±5.52</td>
<td>11.67±4.00</td>
</tr>
</tbody>
</table>

Mean and SD are shown.
Before treatment compared with the 3rd month of therapy: * – p<0.05.
Before treatment compared with the 6th month of therapy: † – p<0.05;
‡ – p<0.001.
The 3rd month of therapy compared with the 6th month of therapy: † – p<0.05.

Table 3. The percentage values of T lymphocytes during therapy in group 2

<table>
<thead>
<tr>
<th>Subpopulation of the lymphocytes</th>
<th>Before treatment n=26</th>
<th>3 months of treatment n=26</th>
<th>6 months of treatment n=25</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3⁺/µl</td>
<td>73.77±9.83</td>
<td>71.46±11.48</td>
<td>70.00±8.32</td>
</tr>
<tr>
<td>CD19⁺/µl</td>
<td>13.38±5.09</td>
<td>12.35±4.87</td>
<td>13.77±5.09</td>
</tr>
<tr>
<td>CD4⁺/µl</td>
<td>48.15±6.66</td>
<td>44.58±6.75</td>
<td>44.18±6.90</td>
</tr>
<tr>
<td>CD8⁺/µl</td>
<td>35.81±7.92</td>
<td>35.77±7.76</td>
<td>33.32±6.81</td>
</tr>
<tr>
<td>CD4⁺:CD8⁺</td>
<td>1.42±0.42</td>
<td>1.32±0.41</td>
<td>1.39±0.37</td>
</tr>
<tr>
<td>CD3⁺/DR%</td>
<td>11.68±3.26</td>
<td>13.34±6.94</td>
<td>11.63±3.76</td>
</tr>
</tbody>
</table>

Mean and SD are shown.
Before treatment compared with the 3rd month of therapy: * – p<0.05.
Before treatment compared with the 6th month of therapy: † – p<0.05.

Table 4. The absolute number of T lymphocytes during therapy in all investigated patients

<table>
<thead>
<tr>
<th>Subpopulation of the lymphocytes</th>
<th>Before treatment n=36</th>
<th>3 months of treatment n=36</th>
<th>6 months of treatment n=35</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3⁺/µl</td>
<td>1720±511</td>
<td>1357±474</td>
<td>1436±543</td>
</tr>
<tr>
<td>CD19⁺/µl</td>
<td>338±175</td>
<td>245±135</td>
<td>281±130</td>
</tr>
<tr>
<td>CD4⁺/µl</td>
<td>1121±325</td>
<td>844±308</td>
<td>900±306</td>
</tr>
<tr>
<td>CD8⁺/µl</td>
<td>829±311</td>
<td>690±276</td>
<td>701±221</td>
</tr>
<tr>
<td>CD3⁺/DR/µl</td>
<td>250±90</td>
<td>250±124</td>
<td>235±93</td>
</tr>
</tbody>
</table>

Mean and SD are shown.
Before treatment compared with the 3rd month of therapy: * – p<0.01;
† – p<0.001.
Before treatment compared with the 6th month of therapy: * – p<0.05;
‡ – p<0.001.

Table 5. The absolute number of T lymphocytes during therapy in group 1

<table>
<thead>
<tr>
<th>Subpopulation of the lymphocytes</th>
<th>Before treatment n=26</th>
<th>3 months of treatment n=26</th>
<th>6 months of treatment n=25</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3⁺/µl</td>
<td>1610±407</td>
<td>1276±410</td>
<td>1435±549</td>
</tr>
<tr>
<td>CD19⁺/µl</td>
<td>359±157</td>
<td>265±170</td>
<td>286±143</td>
</tr>
<tr>
<td>CD4⁺/µl</td>
<td>1078±321</td>
<td>801±279</td>
<td>887±316</td>
</tr>
<tr>
<td>CD8⁺/µl</td>
<td>734±219</td>
<td>662±201</td>
<td>752±206</td>
</tr>
<tr>
<td>CD3⁺/DR/µl</td>
<td>210±80</td>
<td>230±98</td>
<td>248±105</td>
</tr>
</tbody>
</table>

Mean and SD are shown.
Before treatment compared with the 3rd month of therapy: * – p<0.05;
† – p<0.01.

Table 6. The absolute number of T lymphocytes during therapy in group 2

<table>
<thead>
<tr>
<th>Subpopulation of the lymphocytes</th>
<th>Before treatment n=26</th>
<th>3 months of treatment n=26</th>
<th>6 months of treatment n=25</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3⁺/µl</td>
<td>1762±547</td>
<td>1388±501</td>
<td>1436±448</td>
</tr>
<tr>
<td>CD19⁺/µl</td>
<td>330±183</td>
<td>238±122</td>
<td>279±128</td>
</tr>
<tr>
<td>CD4⁺/µl</td>
<td>1137±331</td>
<td>860±321</td>
<td>905±309</td>
</tr>
<tr>
<td>CD8⁺/µl</td>
<td>865±336</td>
<td>700±303</td>
<td>677±228</td>
</tr>
<tr>
<td>CD3⁺/DR/µl</td>
<td>265±90</td>
<td>257±133</td>
<td>230±90</td>
</tr>
</tbody>
</table>

Mean and SD are shown.
Before treatment compared with the 3rd month of therapy: * – p<0.01;
† – p<0.001.
Before treatment compared with the 6th month of therapy: * – p<0.05.

We have proved statistically significant higher percentage of lymphocytes CD3⁺/DR in the investigated group as compared with the control group. Similar result in patients with CHC was recorded by Panasiuk et al. [15]. In the course of 12th week therapy a statistically significant decrease in the percentage of lymphocytes CD19⁺ and CD4⁺ was observed in the investigated group. As well as CD3⁺ and CD4⁺ decrease was also observed in the 24th week of the therapy. What’s more, during the therapy (in 12th and 24th week) statistically significant decrease in the absolute number of lymphocytes CD3⁺, CD19⁺, CD4⁺, CD8⁺ was observed. The observed decrease may depend on IFN-α antiproliferative properties and accumulation of lymphocytes in the liver. The reason of lymphocytes accumulation in the liver in patients with chronic hepatitis and effect of IFN-α on lymphocyte accumulation in the liver are discussed by other authors [16, 17].

Woźniakowska-Gęsicka [18] observed the decrease in the percentage of lymphocytes CD8⁺ after 6 months of the IFN-α therapy. These authors also found the increase of T lymphocytes CD4⁺. Hayata et al. [19] did not observe changes in the percentage of lymphocytes CD4⁺ and CD8⁺ in the peripheral blood in patients with CHC in the course of therapy with IFN-α. These results are different from the
data presented in our paper. The different pattern of T lymphocytes may depend on TP.

It may be assumed that in group 2 the therapy did not help to eliminate HCV infection because no increase in cellular response was observed. The decrease in the percentage and number of lymphocytes CD4+ were observed in the course of the therapy in both groups. Th1 cells are important both in the process of HCV elimination [20, 21] and inducing autoimmune reactions. Piazzola et al. [21] observed decrease in cytokine dependent on Th1 release in patients who did not respond to the IFN-α therapy. Hempel et al. [22] found that lymphocytes both from patients treated with IFN-α who responded well and those who did not respond to the therapy released cytokines Th1 dependent.

TP stimulates Th1 lymphocytes activities [5] so it seems that the therapy with IFN-α and TFX should be taken into consideration in patients with CHC who cannot be treated with combination of IFN-α and Ribavirin.

Conclusions
1. Combination therapy with IFN-α and thymus peptides can be a valuable tool in treating patients with CHC.
2. Lymphocytes T are effector cells of this therapy and during this therapy their redistribution in the organism may return to normal state.

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References