Effect of Hepatitis C Virus Infection on Lymphocytes Proliferation

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جمالية

الشفاء من الإصابة الفايروسية يعتبر بالدرجة الأولى على كفاءة المناعة الخلوية. تم
إجراء هذا البحث لمعرفة كفاءة الخلايا اللمفاوية في السيطرة على الإصابة بفايروس التهاب الكبد

النفط. اخذت عينات دم من مرضى مصابين بالتهاب الكبد الفايروسي نفط سي بعد اجراء

الفحوصات الأولى والنكليكيدية RIBA ELISA وقد قسمت العينات إلى مجموعتين استنادا إلى

درجة تطور المرض حسب فحوص وظائف الكبد والتصوير بالإاموج فوق الصوتية. المجموعة

الأولى تضم 6 مرضى من الحاملين للفايروس (3 منهم من مبتوعي الدم في المركز الوطني لنقل

الدم و3 من مراجعي العيادات الخاصة) وصنفت الإصابة كونها حديثة أو في الطور الحاد أما

المجموعة الثانية فشملت 8 مرضى من مراجعي العيادات الخاصة وصنفت الإصابة كونها قديمة

أو مزمنة.

تم سحب 10 نماذج من اصحاء (HCV negative) لغرض المقارنة.

اجري فحص lymphocyte proliferation باستخدام المنز الدموي النباتي

على المجاميع الثلاثة واستخرج عامل انقسام الخلايا phytoheamagglutinin (PHA)

mitotic index

اجريت العمليات الإحصائية للمقارنة بين المجاميع الثلاثة قبل وبعد أضافة المنز وابعاد معنوية

t–test الفروقات باستخدام فحص

اظهرت النتائج

1- وجود انخفاض معنوي في قابلية الخلايا اللمفاوية للاستجابة لمحفز PHA في مجموعة

المصابين في الطور المزمن مقارنة بالصمام في الطور الحاد للمرض أو من غير

المصابين.

2- اللمحت النتائج إلى أمكانية تحديد درجة تطور الإصابة من خلال معرفة درجة استجابة

الخلايا اللمفاوية للمحفز.
ABSTRACT

Recovery from viral infection depends mainly on the potential of the cellular immunity. This work was performed to assess the efficiency of T-lymphocytes in control hepatitis C virus infection.

Fourteen HCV patients included in this study were categorized to two groups according to disease development as assessed by biochemical tests and ultrasound images. Six patients at acute phase or recent infection group and Eight patients at group of chronic phase. Another 10 HCV negative subjects were included as negative control for comparison. Lymphocyte proliferation test was applied on blood obtained from each subject under study using phytohaemagglutinin (PHA) and the mitotic indices were calculated. Statistical analyses were applied using t-test to assess the significance of the differences between samples tested.

Results showed:
1- Significant decrease was found in the ability of T-lymphocytes from chronic HCV patients to respond to PHA mitogen compared to acute HCV cases or negative controls.
2- The results were promising towards the ability of determining the stage of disease development depending on the degree of response of T-lymphocytes to the mitogen.

INTRODUCTION

Hepatitis C virus (HCV) is a major blood-borne virus that infects over 100 million people worldwide. It is estimated that in less than 20% of HCV-infected individuals the virus is cleared spontaneously, while in the majority of patients the virus persists and causes chronic hepatitis that may lead to end-stage liver diseases requiring liver transplantation (1). The mechanisms underlying different outcomes of infection are not clear at this time. The host immune responses, including innate immunity and adaptive immunity, play a critical role in determining the outcome of viral infection, as well as in the nature and extent of liver cell injury during HCV infection (2,3). Since the rate of persistence for HCV is much higher than other hepatitis viruses, for example, hepatitis B virus (HBV) that persists in only less than 10% of immunocompetent adults who are infected (4), HCV appears to be more successful than many other viruses in terms of evading the protective immunity of the host. However, little is known regarding the exact reasons for the failure of the host immune system in fighting HCV.

Innate immunity is an important first line of defense against infection, quickly responding to potential attacks by pathogens. One of its arm is the lymphocytic cells (natural killer [NK] and natural killer T [NKT] cells) which can kill pathogens nonspecifically. However, the role
of NK cells in human HBV and HCV infections is less clear because of a lack of suitable small-animal models (5).

In vitro culture experiments showed that NK cells inhibit HCV replication in human hepatoma cells via an IFN–dependent mechanism. A retrospective study revealed that individuals with a genetic predisposition to enhanced NK function had greater chances of spontaneously clearing HCV during acute infection (6) suggesting that NK cells play an important role in early antiviral defenses against HCV. In contrast, HCV can escape the antiviral response of NK cells by inhibiting NK cell function, and this results in chronic HCV infection in the majority of patients (7-9). HCV may have a pervasive influence on the general T cell immunity of infected hosts, which is not limited to HCV-specific T cells.

A critical factor for the development and differentiation of T cells into functional memory and effector cells is the stimulation that they receive during the primary response (10). Stimulation of T cells from virally infected individuals with the lectin phytohemagglutinin (PHA) found to increase cytokines production (11) and to enhance the anti-viral effect of certain antiviral agents (12) and to distinguish between patients at different stages of certain viral diseases (13).

However, in order to understand the apparent weak or abnormal lymphocytes immunity to HCV, it is important to investigate the efficacy of lymphocyte response leading to the antiviral innate and adaptive immunity.

**MATERIALS AND METHODS**

Twenty four individuals were included in this study divided into three groups. Anti-HCV positive patients were divided into two groups according to biochemical tests and ultrasound pictures done by internal medicine specialists.

Group(1): Includes six anti-HCV positive individuals (3 individuals were blood donors from the national center for blood transfusion and 3 individuals from special clinics ) diagnosed as acute or recent infections.

Group(2): Includes eight anti-HCV positive patients (visitors of special clinics) diagnosed as chronic infections.

Group (3): includes ten anti-HCV negatives. This group used as control for comparison.

Venous blood samples (5 ml) were obtained from each subject studied using disposable syringes. Each sample was distributed into two tubes: 1ml of blood was added into tube containing 20ul of heparin to be used in the Mitotic index assay. The remaining of the blood sample was left to clot and sera were collected and kept in freezer to be used in the virological and serological assays.
Mitotic index Assay: The procedure of (14) was followed with slight modification using Phytohemagglutinin (PHA) as mitogen (supplied by Iraqi center for cancer researches and medical genetics):

1) 0.3 ml of heparinized blood was inoculated into two venoject tubes contain 4ml of culture medium for each. 0.3ml of PHA is added to one of them and labeled as (withPHA), the other tube is not treated with PHA and labeled as (without PHA).
2) The tubes are incubated at 37C for 72 hrs with gentle shaking two times daily.
3) The cultures are transferred to centrifuge tubes and spunned at 1500 rpm for 10 min.
4) The supernatant is discarded and 10ml of pre-warmed (0.075 M) KCl is added and mixed thoroughly.
5) The tubes are incubated at 37C for 50 min.
6) The tubes are centrifuged at 1500 rpm for 10 min. and the supernatant is discarded.
7) 5 ml of freshly prepared fixative (3 parts of methanol to 1 part of acetic acid) is added drop by drop and shaken gently.
8) Steps 6 and 7 are repeated at least twice more.
9) The tubes are spunned at 1500 rpm for 10 min.
10) Cells pellet is resuspended in a small volume of fresh fixative and dropped (from about 50 cm height) onto a highly-cleaned microscopic slide and allow to dry in air.
11) Slides are stained for 15 min. with Giemsa’s stain, wash with tap water, dry, and examine for the presence of lymphoblast cells.
12) At least 500 cells are counted and the result is recorded as:

\[
\text{Mitotic index} = \frac{\text{No. of Lymphoblast}}{\text{Total no. of lymphocytes counted}} \times 100
\]

Hepatitis C virus detection was done by using the third generation bio Elisa HCV screening kit (supplied by biokit, Spain). Anti- HCV positive cases were confirmed by using recombinant immunoblot assay (RIBA). recomBlot HCV IgG (supplied by Microgen, Germany).

T-test for independent samples (SPSS v17) was used for comparing means of mitotic indices of anti-HCV positive and negative groups with confidence interval (95%). For PHA-treated lymphocytes, differences in the mitotic indices of lymphocytes before and after treatment with PHA for anti-HCV positive and negative groups were calculated, and then a comparison between the two means was made.
RESULTS AND DISCUSSION

Table -1: Mitotic indices of Anti- HCV positive & Negative groups before and after treatment with PHA.

<table>
<thead>
<tr>
<th></th>
<th>Anti- HCV positive</th>
<th>Anti-HCV negatives</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acute (or recent)</td>
<td>Chronic</td>
</tr>
<tr>
<td>PHA treated</td>
<td>PHA untreated</td>
<td>PHA treated</td>
</tr>
<tr>
<td>52</td>
<td>12</td>
<td>32</td>
</tr>
<tr>
<td>58</td>
<td>8</td>
<td>30</td>
</tr>
<tr>
<td>42</td>
<td>10</td>
<td>43</td>
</tr>
<tr>
<td>54</td>
<td>8</td>
<td>38</td>
</tr>
<tr>
<td>25</td>
<td>12</td>
<td>22</td>
</tr>
<tr>
<td>44</td>
<td>11</td>
<td>35</td>
</tr>
<tr>
<td>34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means of the mitotic indices (table 1) were calculated (table 2) and it is clear that anti-HCV positive samples, either of acute or chronic cases, have higher means than that of negative group before treatment with the mitogen.

The difference in mitotic index of samples before and after treatment with PHA reveals that chronic HCV cases had the lesser mean within the groups suggesting that HCV infection is implicated in this process.

Table -2: Calculated means of mitotic indices of the tested groups.

<table>
<thead>
<tr>
<th>Anti-HCV</th>
<th>PHA Treatment</th>
<th>Mean</th>
<th>Std. deviation</th>
<th>Std.error mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute (6 subjects)</td>
<td>Treated</td>
<td>45.83</td>
<td>11.87</td>
<td>4.84</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td>10.16</td>
<td>1.83</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>Difference between Treated &amp; untreated</td>
<td>35.66</td>
<td>13.155</td>
<td>5.37</td>
</tr>
<tr>
<td>Chronic (8 subjects)</td>
<td>Treated</td>
<td>33.75</td>
<td>6.158</td>
<td>2.177</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td>18.37</td>
<td>4.68</td>
<td>1.657</td>
</tr>
<tr>
<td></td>
<td>Difference between Treated &amp; untreated</td>
<td>15.37</td>
<td>1.846</td>
<td>0.652</td>
</tr>
<tr>
<td>Negative (10 subjects) control</td>
<td>Treated</td>
<td>46.2</td>
<td>9.761</td>
<td>3.086</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td>2.8</td>
<td>2.859</td>
<td>0.904</td>
</tr>
<tr>
<td></td>
<td>Difference between Treated &amp; untreated</td>
<td>43.40</td>
<td>10.905</td>
<td>3.448</td>
</tr>
</tbody>
</table>
The significance of these differences was tested (as shown in table 3). Table 3 shows that untreated anti-HCV positive samples have significantly higher mitotic index than anti-HCV negative group. Moreover, untreated HCV chronic samples showed significant increase in mitotic index compared to samples of acute cases. These results clearly indicates that HCV infection stimulate lymphocytes proliferation. No significant difference between untreated samples of acute and control groups reveals that HCV infection may requires along period of time to negatively affect lymphocytes proliferation capacity.

The significance of differences within each group before and after treatment indicates that PHA can stimulate lymphocytes proliferation in HCV positive samples, as well as in negative group, although with less ability in chronic group (mean difference is 15.37 as compared with 35.66 and 43.4 for acute and control groups, respectively. This difference indicates that the lymphocytes of chronic HCV patients lost much of their potential to respond to the stimulator.

After treatment with the mitogen, significant decrease in mitotic index was found in HCV acute cases compared with the control group. Comparison of the mitotic index between chronic HCV with acute HCV groups showed significant decrease in the index of chronic group. This means that HCV disease progression from acute to chronic stage simultaneous with decreased capacity of lymphocytes to proliferate in response to stimulators.

Table -3: t- test for the equality of means of mitotic indices of the tested groups.

<table>
<thead>
<tr>
<th>Tested groups</th>
<th>T</th>
<th>Sig.(2-Tailed)</th>
<th>Mean Difference</th>
<th>Std.error Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute (untreated) VS Acute (treated)</td>
<td>7.272</td>
<td>.000</td>
<td>35.66</td>
<td>4.904</td>
</tr>
<tr>
<td>Acute (untreated) VS chronic (untreated)</td>
<td>4.03</td>
<td>0.002</td>
<td>_8.208</td>
<td>2.036</td>
</tr>
<tr>
<td>Acute (untreated) VS control (untreated)</td>
<td>5.613</td>
<td>0.00</td>
<td>7.366</td>
<td>1.312</td>
</tr>
<tr>
<td>Chronic (untreated) VS chronic (treated)</td>
<td>5.618</td>
<td>0.00</td>
<td>15.375</td>
<td>2.736</td>
</tr>
<tr>
<td>Chronic (untreated) VS control (untreated)</td>
<td>8.708</td>
<td>0.00</td>
<td>15.575</td>
<td>1.788</td>
</tr>
<tr>
<td>Control (untreated) VS control (treated)</td>
<td>13.492</td>
<td>0.00</td>
<td>43.40</td>
<td>3.216</td>
</tr>
<tr>
<td>Acute (treated) VS chronic (treated)</td>
<td>2.488</td>
<td>0.29</td>
<td>12.083</td>
<td>4.856</td>
</tr>
<tr>
<td>Acute (treated) VS control (treated)</td>
<td>0.067</td>
<td>0.947</td>
<td>_0.366</td>
<td>5.455</td>
</tr>
<tr>
<td>Chronic (treated) VS control (treated)</td>
<td>_3.13</td>
<td>0.006</td>
<td>_12.45</td>
<td>3.974</td>
</tr>
</tbody>
</table>
The mean of differences between treated and untreated samples (as shown in table 4) of each group found to be significant and the difference between chronic and acute cases was less than that between chronic and control groups.

Table -4: Results of t-test for the differences in mitotic indices between anti-HCV positive and anti-HCV negative groups

<table>
<thead>
<tr>
<th>Mean of differences between treated &amp; Untreated samples</th>
<th>t- test for equality of means</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
</tr>
<tr>
<td>Acute VS chronic</td>
<td>4.365</td>
</tr>
<tr>
<td>Acute VS Control</td>
<td>1.274</td>
</tr>
<tr>
<td>Chronic VS Control</td>
<td>7.144</td>
</tr>
</tbody>
</table>

Since disease persistence and progression to chronicity strongly associated with the efficiency of the cellular response, mitogen-stimulated PBML widely used in assessing the functions and efficiency of T-cells in controlling viral infections. PHA- stimulated lymphocytes successfully used in T-cell cytotoxicity test for distinguishing between the different stages of HIV infections (13).

Accumulating evidence suggests that dendritic cells (DCs) are susceptible to HCV infection (15-18). Several groups have reported dysfunction of DCs that may potentially affect adaptive immunity in patients with persistent HCV infection. These include impaired allostimulatory abilities to CD4 T cells (15,17, 19-21), defects in responding to maturation stimuli (20), as well as impaired ability to secrete IL-12, a cytokine important for the development of CD4 helper T cell responses (21-22).

Although HCV persists in the majority of infected individuals, a small fraction of patients can successfully clear the infecting virus. The number of cases with self-limited HCV infection that have been carefully studied is relatively small, such studies usually reveal a vigorous HCV-specific T cell response, including CD4 T cell response (23-26) and CD8 T cell response (24,25,27-29). These responses were detected early during the acute phase (24,25) and sustained for many years after the clearance of HCV (24). They were usually broadly targeted at multiple epitopes restricted by different MHC molecules, without a dominant epitope (29). Significantly high mitotic index mean of samples from acute
HCV cases in this study which reflects the high potential of T cells to respond to stimulators strongly support these findings. Moreover, high numbers of PHA-activated lymphocytes of acute phase samples reveals that HCV infection, for unknown reasons, does not rapidly affect the proliferation capacity of T cells inspite that it considered targets for HCV replication.

In contrast, patients with chronic HCV infection usually have weak or defected T cell responses against HCV, as indicated by the low response to PHA stimulation compared to samples of acute cases or controls. Previous studies reported low frequencies for the specific T cells (29,30), short-lived responses (31,32), narrowly targeted epitopes (29), as well as defects in the effector functions of the specific T cells (33,34) which support our findings. Although the mechanism for HCV to evade host immune responses and establish chronic infection is still poorly understood, accumulating data indicate that HCV may play an active role in attenuating host adaptive immunity to benefit its persistence. In HCV chronically-infected patients treated with IFN, increased T cell immunity after IFN therapy has been demonstrated for HCV-specific CD4 T cells (35-37) and CD8 T cells (38,39) which reflect the initial role of T cells in eliminating or persistence of HCV infection.

Taken together, these studies strongly suggest that the host T cell responses are a key factor in determining the outcome of HCV infection.

In this study, HCV chronicity found to be associated with low T-lymphocytes response to stimulation in comparison with acute or control samples. Upon these findings, further studies needed to determine the exact mitotic index value corresponding to the stage of HCV disease in order to manage disease treatment and control in order to replace the sever, invasive, and complicated technique, liver biopsy, by simple, ease, less invasive and cost effective one.

REFERENCES


