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Necessity for modern methods of diagnosing Hepatitis C, especially when overlapping with secondary infection

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Abstract

In this study, we focused on the antimicrobial peptides and T-lymphocyte clusters indicating the immune system ability to react violently leading to reduce the organism sensitivity to bacteria tested in 87 individuals with chronic hepatitis C. All patients were divided into two groups: with chronic hepatitis C, and the group in which hepatitis C overlaps with secondary infection leading to pneumonia. Endotoxin and lipopolysaccharide-binding protein (LBP) were determined using the ELISA technique. Determination of the clusters of differentiation (CD) carried out by indirect immunofluorescence reaction, while the circulating immune clusters (CIC) identified by method of sedimentation with a 3.5% solution of polyethylene glycol. Statistical processing of the results carried out using the Wilkinson U-test (Mann - Whitney). In the group of patients with hepatitis C, CD25+ was nearly halved, while in group with hepatitis aggravated by pneumonia this value lowered approximately three times. CD25+ indicator in II group was even 1.4 times less than in the group without pneumonia. Defensin levels were significantly higher in the I group, where endotoxin raises up to 24.4 vs normal levels. In the II group, aggravated by pneumonia, endotoxin elevated even up to 57.7 IU/ml, the same direction changed defensin concentration. The results of this study show, that instead of standard tests for liver damage: bilirubin, AST and γ-glutamyl transpeptidase, it is much more expedient to use the antimicrobial peptides defensin and LBP, which are more informative in the diagnosis of hepatitis C, especially when it overlaps with secondary infection.

Keywords: Circulating Immune Complexes; Defensin; Endotoxin; Lipopolysaccharide Binding Protein

1. Introduction

All living organisms are threatened by numbers of microorganisms seeking to exploit the host cells components, which is why most cells largely produce natural antibiotic-like molecules, namely antimicrobal peptides. Synthesis of antimicrobal peptides is pivotal moment in host defense: they kill or inhibit the growth of foreign presenters by binding to bacterial membranes either to disrupt the membrane or to enter the bacterium and inhibit intracellular function. Zhang (2016) state, that microbes produce a variety of anti-microbal peptides to reduce the growth of other microorganisms, and these peptides from microbes can be synthesized by assistance of non-ribosomal peptide synthase [1]. Some antimicrobal peptides modulate host immunity by recruiting or activating immunocytes, they protect the body from a wide range of gram-positive and gram-negative microorganisms, fungi and viruses. Ergo, endogenous antimicrobial peptides are non-specific factors of the body immune defense, which help to neutralize endotoxins, as well as stimulate immune-protective mechanisms. The antimicrobal peptides have selective antimicrobial activity against organismsmodulating host cellular immunity and enhancing host defenses due to their electronic interaction with negatively charged membranes of gram-negative bacteria, and this is important for the maintenance of many aspects of health.

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Hepatitis C is still a major health burden, because about 70% of infected people progress to chronic infection, and aspects concerning the immune defense during the development and chronic hepatitis C remain still unstudied [2]. Approximately 170 million people in the world are infected with hepatitis C virus (HCV). HCV is an enveloped virus infecting humans only, it primarily targets liver cells. HCV evades innate and adaptive immunity and causes chronic infections in 70% of cases; without treatment, it leads to cirrhosis of the liver in 20% of cases, and some of them progress to hepatocellular carcinoma [3].

The aims of this work were to assess whether liver infection HCV is associated with the antimicrobal peptides availability in the blood, and the relationship of antimicrobial peptides with immune system indicators in the development of chronic viral hepatitis C. In order to examine the mechanisms by which hepatitis HCV developed, we analyzed the changes of immune system markers: the clusters of differentiation CD3+, CD4+, CD8+, CD14+, CD16+, CD25+ and along with them, T- and B-lymphocytes, circulating immune complexes, antimicrobal peptides in the blood of patients with viral hepatitis HCV and hepatitis C complicated by pneumonia. Among CD4+ T cell subsets, lymphocytes expressing CD25+, namely T regulatory cells, play a critical role in controlling chronic evolution of HCV mediated liver diseases [4]. Cabrera (2004) affirmed, that both LBP and CD14+ are associated with systemic inflammation. We were interested in whether CD4+, as well as CD4+/CD8+ and CD4+/CD25+ ratios were associated with the development of chronic hepatitis C [5].

LBP is a recently identified hepatic secretory protein potentially involved in the pathogenesis of sepsis, capable of binding the bacterial cell wall product, endotoxin, and directing it to its cellular receptor, CD14+, and by this way lipopolysaccharide of microorganism interacts with the innate immune system, facilitated through LBP of host and the co-receptor CD14+ [6].

Taking into account, that in the pathogenesis of hepatitis caused by HCV, the leading role belongs to endotoxins of gram-negative bacteria, we investigated whe-ther circulating levels of LBP and CD14+ are associated with the severity of viral he-patitis C identified by raised levels of endotoxins.

2. Material and methods

2.1. Experimental Design

In order to achieve the aim, the blood of 87 patients aged 17-38 was examined. All identified patients were divided into two groups. The first group of patients comprised 45 people in whose blood chronic hepatitis C virus was detected, the second group (n = 42) consisted of patients in whose blood the HCV was complicated by a bacterial infection (pneumonia). The control group consisted of 20 healthy people. The diagnosis of hepatitis C was confirmed based on the classification proposed by the World Congress of Gastroenterology (Los Angeles, 1994).

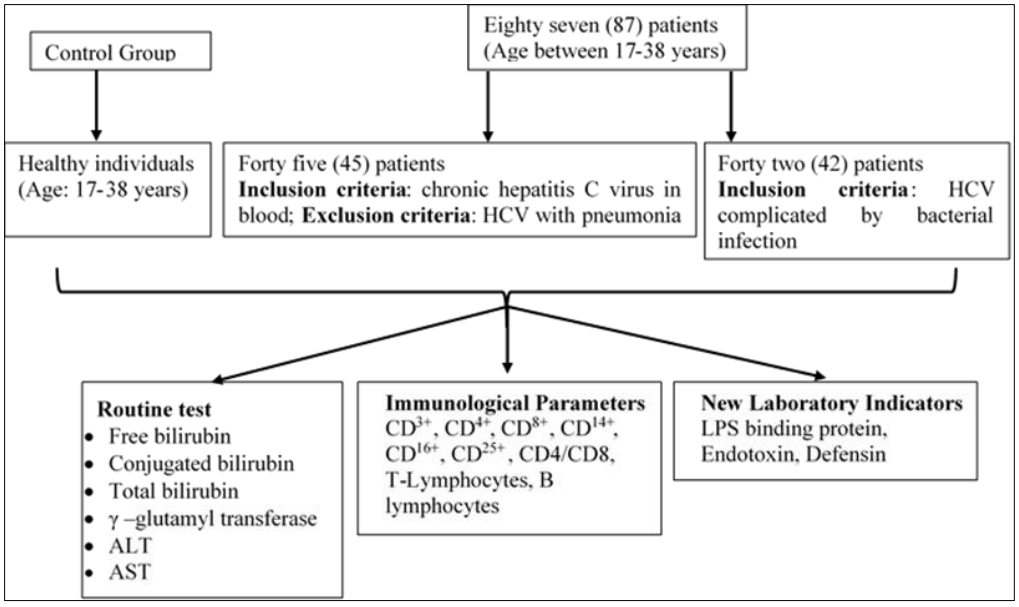

Figure 1 Work arrangements

2.2. Biochemical Assay

As biochemical markers of hepatitis, along with total, free and conjugated bilirubin, 3 enzymatic indicators of liver function: ALT, AST, as well as gamma-glut amyl Tran's peptidase were studied. Bound and free bilirubin were determined by the Yendrashik method, ALT and AST determined by the Reitman-Frenkel method, and the concentration of total protein and gamma-glut amyl Trans peptidase determined by the German set "Diasys".

2.3. Immunological assay

Determination of endotoxins and LBP carried out by enzyme-linked immunosorbent assay (ELISA) using a set of "Hy Cult Biotechnology" (Holland) based on the solid-phase "sandwich" principle.

In order to study the cellular and humoral aspects of the immunity, the composition of the lymphocyte antigens CD^{3+} (T-cells), CD^{4+} (helper T-cells), CD^{8+} (suppressor/cytotoxic T-cells), CD^{14+} (monocyte/macrophage), CD^{16+}, CD^{25+} (the α-chain of the interleukin 2 receptor on activated T cells) was determined by an indirect single immunofluorescence staining, for which a BD FACS ™ Brand flow cytometer was used. A fluorochrome-conjugated monoclonal antibody solution was added to 100 µl of whole blood in a test tube and incubated for 20 minutes in the dark at room temperature (20 to 25 ° C). Then, 2 ml of BD FACS Lysing Solution was added, vortexed and incubated for 10 minutes in the dark. To remove the supernatant, the resulting mixture was centrifuged at 300 g for 5 minutes, then washed with 3 ml of BD Cell WASH solution and centrifuged again to remove the supernatant at 200 g for 5 minutes. At the end, 0.5 ml of buffer was added, mixed thoroughly, & the resulting solution was analyzed immediately.

The determination of CIC based on method of precipitation with a 3.5% solution of polyethylene glycol method.

2.4. Statistical analysis

Statistical processing of the obtained results was carried out by determining the Wilkinson's U-test (Mann–Whitney). $P < 0.05$ was accepted as reliable.

3. Results

Table 1 Routine test indicators of the control group and patients with chronic hepatitis C infection

Routine test results	Control group (n=20)	Hepatitis C (n=45)	Hepatitis C with secondary infection (n=42)
Free bilirubin, µmol/l	12.74 ±0.62	15.92 ±0.68**	14.91 ±0.76
Conjugated bilirubin, µmol/l	3.94 ±0.15	4.92 ±0.25*	4.52 ±0.36
Total bilirubin, µmol/l	16.3±0.7	18.3±0.7	17.2±0.4
γ –glutamyl transferase, İU/l	15.24 ±1.16	17.24 ±0.83	28.61 ±1.07***
AST, mmol/l	0.41 ±0.02	0.47 ±0.03	0.74 ±0.08**
ALT, mmol/l	0.51 ±0.03	1.16 ±0.12***	1.16 ±0.07***

Note: *p <0.05;**- p <0.01;***-p < 0.001 statistically significant difference compared to control group

All obtained biochemical data, as well as data from patients with chronic hepatitis C complicated by pneumonia, are represented in tables given below. As follows from the table 1, the main indicators of liver activity, namely the total blood bilirubin and its fractions, including free bilirubn fraction, as well as AlT, AST were slightly increased in the 1st group and the same is seen in the second group. These data correlated with the severity of hepatitis and its clinical manifestations. In both cases ALT/AST ratio were higher than 1 (ALT/AST>1), what indicates that the pathological process affects the cell membrane ragher than the organell membrane, because othervise this ratio had to be less than 1 (ALT/AST<1). Indicators ALT and AST along with gamma-glut amyl transpeptidase are used as typical indicators of the severity of the liver viral disease, and a noticeable increase in them indicates unfavorable prognosis for the disease development. It is acknown that AST sharp increase characterizes the degree of mitochondrial damage, and complication of disease leading to hepatocytes necrosis, but we did not observe any dramatic raise of AST. Note that in the second group, the activity of gamma-glutamyl transpeptidase increased 1.9 times, which also indicates the activation of hepatocyte cellular membrane damage. Gamma-glutamyl transpeptidase is a membrane-associated enzyme of hepatocytes, ergo its increase means damage of the cytoplasmic membrane of liver cells with subsequent leaching of

the cytoplasmic content, namely ALT into the circulating blood. The accession of a bacterial infection to the liver damage constitutes the most widespread type of complication of the course of the disease, which is why the raise in AST and especially, γ-glutamyl transpeptidase may be accepted as result of this kind complication. The appearance of the most noticeable changes in AST and gamma-glutamyl transpeptidase in the second group of patients confirms the fact of additional damage to the liver by the attached infection.

As follows from table 2, in I and II groups we found noticeable changes in the indices of immune system versus control group.

Table 2 Indicators of immune system state of the control group and patients with chronic hepatitis C infection measured by new methods

Immunological parameters	Control group (n=20)	Hepatitis C (n=45)	Hepatitis C with secondary infection (n=42)
CİC, İU	103.40 ±7.00	130.40 ±8.68	169.63 ±9.15***
CD25 %	20.3 ±1.4	10.67 ±1.08***	7.64 ±0.89***
CD4, %	32.10 ±1.38	26.65 ±1.49**	27.71 ±0.92*
CD14, %	16.05 ± 0.62	17.06 ±0.54	18.16 ±0.54*
CD3, %	62.4 ±1.52	58.07 ±1.36	59.71 ±1.41
CD8, %	29.75 ±7.10	28.34 ±1.59	27.39 ±1.62
CD16, %	13.25 ±1.08	12.85 ±0.73	12.26 ±0.72
CD4 / CD8, %	1.08±0.03	0.94±0.02	1.01±0.02
T-lymphocyte, %	52,42 ±1.93	61.54 ±1.83**	57.6 ±1.82
B-lymphocyte, %	33.35 ±2.74	19.84 ±1.70***	29.52 ±1.4

Note: *p <0.05;**- p <0.01;***-p < 0.001 statistically significant difference compared to control group

Despite the fact that percentage of T-lymphocytes has not lowered in the blood of I group patients, nonetheless the deficiency of the T-lymphocytic protection system becomes clear by trend to decrease in cluster of differentiation 3 (CD3+), significant decrease in the patient blood indicators CD4+. As can be seen from the table 2, negligible changes in CD3, CD8 and CD16 clusters percentage in the blood do not deserve attention. We did not find any significant change in the CD8 + count, but only a downward trend in CD8 +. Thus, a decline in indicator CD4+and trend to decline CD8+ may indicate a deficiency in the immune system, namely the insufficiency in the helper effect, as well as in expansion and differentiation of resting T-cells into cytotoxic effector cells [6, 7].

In I group of patients, a significant decrease in the number of B-lymphocytes was also noted. A parallel decrease in CD16+ and CD25+with a simultaneous raise in the number of T-lymphocytes indicates an imbalance in a specific cell-mediated immunity, but with less suppression of T-cell defense system, because observed activation of T-cell lineage of immune system (especially in I group) may be an indicator of defense of patients with chronic HCV infection rather via T-cells, than through B-cells. In this regard, it is appropriate to note, that according to most scientists, the decisive factor in the development of hepatitis C is a violation of the T-cell component of the immune defense [8]. It is acknown, that the main function of B-lymphocytes is the production of corresponding antibodies after appropriate antigen-antibody type reaction resulting in transformation into plasma cells. However, for the implementation of the processes mentioned above, presence of a CD4+ cell component is required. We have already noted, that a marked decrease in the CD4+ levels in both groups of examined patients was found. Consequently, in blood of examined patients HCV specific antibodies, even being produced, are not able to bind with the required amount of virus, because CD4+ in their organism is reduced [9].

Determination of the CIC levels in the blood show their increase in both groups of patients, especially in the second group (in group I this indicator was 130.40 ± 8.68 IU, in II 169.63 ± 9.15 IU ($p < 0.001$) versus control group = 103.40 ± 7.00 IU). An increase in CIC may indicate an absorption of antigens by specific antibodies. This may also be an indicator of blocking complement resulting in the persistence of the virus, which in turn leads to prolongation of the chronic process in hepatocytes [10].

Table 3 New laboratory indicators in the control group and patients with chronic hepatitis C infection

Antimicrobial peptides along with endotoxin	Control group (n=20)	Hepatitis C (n=45)	Hepatitis C with secondary infection (n=42)
Lipopolysaccharide-binding protein, mq/ml	23.6 ±4.2	138.7± 8.9***	443.6± 29.6***
Endotoxin, İU/ml	0.10 ±0.01	24.4 ±2.3***	57.7 ±5.2***
Defensin, nq/ml	38.6± 3.8	297.0± 17.5***	1467.3± 27.7***

Note: * - $p < 0.05$, ** - $p < 0.01$, and *** - $p < 0.001$ statistically significant difference compared to control group

When HCV infection, the content of endotoxin in the first group was = 24.4 ± 2.3 IU / ml, in group II = 57.7 ± 5.2 IU / ml, whereas in the control group this indicator was 0.10 ± 0.01 IU / ml (see Table 3).It is known that in chronic viral hepatitis C, the endotoxin levels in the blood may raise several times, and this phenomenon indicates increase of endotoxin aggression in the body, which is primarily due to impaired neutralizing function of liver, increased intestinal permeability and the syndrome of bacterial infection [11].

The excitation and progression of the immunological inflammatory process through endotoxin occurs via the various mechanisms. It is necessary to keep in mind, that endotoxin stimulates the appearance of defensins [12; 13].

According to our study, in the I group the defensin levels increased 7.7 times (297.0 ± 17.5 ng / ml) versus control (38.6 ± 3.8 ng / ml), whereas in the II group, compared to the I group this indicator increased already 5 times (1467.3 ± 27.7 ng / ml). On the one hand, defensins have obvious bactericidal effect, on the other hand they are able to deepen the alteration in the inflammatory focus by damaging the host cells in the body [14]. It is known that defensins play the role of opsonins, even chemokines. Thus, they cause accumulation of certain substances, such as immature dendrites, monocytes and T-lymphocytes, in the inflammation zone. Numerous effects of defensin have been established, such as increased proliferation and maturation of various immune system cells, stimulation of expression adhesive molecules and molecules of the histocompatibility complex from different cells, and facilitation of cytokines activity causing the inflammatory process in any cell, and so on [15; 16]. As follows from the research results, defensins are fairly informative markers of the severity of the inflammatory process in HCV infection. In order to analyse the liver response on inflammatory process, we also determined the content of LBP. Need to mark, that the LBP levels correlated with the severity of the infectious process in the liver. It was found, that the highest levels of this indicator appears among the patients of the second group; in this group the LBP levels increased 18.8 times (443.6 ± 29.6 ng / ml) versus control. For comparison, the same indicator increased 5.9 times (138.7 ± 8.9ng / ml) in group I. On the one hand, an increase in the level of LBP is a marker of endotoxemia severity, but on the other hand, this may indicate an increase in the immune defense against endotoxins, because LBP is capable of binding the bacterial cell wall product - endotoxin, and directing it to its cellular receptor, namely CD^{14+} for detoxification [17], which is why with the development of a bacterial infection, the levels LBP in the blood start to raise rapidly. It is possible that this fact may allow the definition of LBP protein in the blood to be used to quantify endotoxemia, and as a valuable marker of infection in the body. Analyzing the constituents of the viral hepatitis C, concentration of LBP was found to be significantly higher in patients with viral hepatitis, and much more higher in patients with hepatitis C complicated by pneumonia. When chronic hepatitis C, the endotoxin content in the first group was 24.4 ± 2.3 IU / ml, in group II - 57.7 ± 5.2 IU / ml versus 0.10 ± 0.01 IU / ml in the control group ($p < 0.001$, statistically significant difference compared to control group).It is known that in inflammation the endotoxin content in the blood can increase several times, ergo we assume, that endotoxin aggression increased mainly in the blood of patients, which have already been primarily associated with impaired hepatic neutralizing function, increased intestinal permeability and the syndrome of bacterial infection [18]. The excitation and progression of the immunological inflammatory process through endotoxin occurs via the inclusion of various mechanisms [19; 20].

Since our study shows sharp raise in defensins and LBP, we conclude that this indicates the specialisation of the immune system mainly in this direction of defence [21; 22; 23].In general, the activity of the pathological process in the liver directly influenced on the antimicrobial peptide levels determined in blood plasma, and the indicators deteriorated most dramatically in the group of patients with chronic hepatitis C complicated by pneumonia.

4. Discussion

As can be clearly seen from table 1, total bilirubin does not raise significantly in patients with chronic hepatitis HCV, neither in the first, nor in the second group. This indicates ability of glucuronyl transferase reaction to be sufficiently

active in such patients. Nevertheless, there was a trend for increase of total bilirubin, free and conjugated bilirubin in both groups. Interestingly, the trend towards an increase in the levels of free and conjugated bilirubin was more pronounced in group I, than in the second one. It can be assumed that this indicator should be higher in patients with complicated development of hepatitis C. However, a lesser tendency to an increase in bilirubin in group II may be a consequence of anemia, usually accompanying an infection complicated by pneumonia [24]. However, our analyzes found, that bilirubin did not raise to the levelgreater than 25 μmol/l/l, ergo jaundice did not appear in them and the microsomal activity of patients of both groups with hepatitis C may be taken as satisfactorily working with downward trend.

ALT increased significantly in both groups with hepatitis C ($p<0.001$). In the first group, the ALT level had already tripled; and, undoubtedly, this indicates damage to the outer membrane of heparocytes in patients with viral hepatitis C with the leaching part of the intracellular ALT into the blood. Accession of pneumonia did not increase this indicator, and this phenomenon can be considered as evidence of ALT washing out exclusively from hepatocytes under the action of HCV virus, but not in any way the infection associated with pneumonia.

AST did not significantly increase in the first group, which means that HCV infection does not destroy the mitochondria of hepatocytes, otherwise AST would raise as much as ALT, even more. But second group showed statistically significant increase of AST, which may indicate deepening of infection with damage mitochondria in patients with hepatitis C complicated by pneumonia. May be this AST is washed out not from hepatocytes, but from lung tissue?

Activity of γ –glutamyl transferase trends to increase in the first group & significantly raised in group of patients with hepatitis C after accession of secondary infection ($p<0.001$). Taking into account that γ –glutamyl transferase is a membrane enzyme responsible for transportation of amino acids into the liver cells, we can conclude, that in the first group there is no need to activate the transport of amino acids into hepatocytes, but in the second group this process has been activated. It is possible, that these amino acids are released from damaged lung tissue due to pneumonia. An increase in the level of γ-glutamyl transferase additionally confirms the fact of damage to the membrane of hepatocytes, and to a greater extent - in patients with disease complicated by pneumonia. So, the first reason for the increase in this enzyme activity in the blood may be damage to the liver membrane, but the second reason may be the activation of the transport to the liver amino acids released from the lungs damaged by infection.

We also analyzed clusters of differentiation (CD), expressed on the surface of lymphocytes, and changes in the percentage of lymphocytes in both groups of patients. Clusters of differentiation are sort into: CD3+ (T cells), CD4+ (T helper/regulatory T cells), CD8+ (cytotoxic T cells) and so on [25]. A downward trend in CD3+ in both groups should indicate a decrease in the number of T-lymphocytes, but we found an increase in T-lymphocytes in the blood of patients of the first group. CD3+is a surface marker specific to all cells of the T-lymphocyte subpopulation. By function, it belongs to the family of proteins, which form a membrane signal transduction complex associated with the T-cell receptor. It is a T cell co-receptor, that activates both the cytotoxic T cell (CD8+ naive T cells) and T helper cells (CD4+ naive T cells). Since the CD3 + protein complex binds to the T-cell receptor to generate an activation signal in T-lymphocytes, it can be concluded that with fewer CD3 + clusters, this ability of T-lymphocytes will also trend to decrease, even if the number of T-lymphocytes increases.

CD4 + is a cluster of differentiation, that occurs on the surface of helper T cells, which play an important role in the immune system, especially in the adaptive immune system. As the name suggests, they release cytokines to help activate other immune cells. A lack of CD4+ cells usually leads to frequent infections, which is why we conclude that significantly reduced CD4 + levels in the second group indicates a deficiency of this lineage in the immune system in patients with hepatitis C, and makes these patients susceptible to infection. The ratio CD4+/CD8+ is also used to determine ability of immune system to defend the host cells from foreign presenters. The ratio greater than 1, or even between 1.5 and 2.5 are generally considered normal; depletion of CD4 + T-cell populations in combination with progressive impairment of cellular immunity increases susceptibility to opportunistic infections [26]. We found CD4+/CD8+ ratio lowered less than 1 in group I, but not in group II, because CD4+ was higher in the I group than in the group II.

CD8+ does not change significantly either in I, or in the II group of patients. It is acknown that CD8+ is a transmembrane glycoprotein that serves as a co-receptor for the T-cell receptor, and plays a role in T cell signaling and aiding with cytotoxic T cell antigen interactions; CD8+ binds to a complex molecule, but is specific for the major histocompatibility complex I [27]. Turning to our results, we have to say that these functions are not impaired in the patients examined. Resting naive CD8+ T cells react to pathogens by massive expansion and differentiation into cytotoxic effector cells that migrate anywhere to clear body from infection. The initial interaction with antigen-presenting cells in the central lymphoid part leads to differentiation of immune cells producing sufficient numbers of effectors. According to our data, this lineage of immune system was within normal ranges in both groups of patients with hepatitis C.

CD14+ is a glycolipid membrane glycoprotein, that is expressed on cells of the myelomonocyte lineage, such as monocytes, macrophages, and some granulocytes. CD14+ is an activator of innate immune cells. CD14+ is expressed in a variety of hematopoietic cells and has a range of biological activity including differentiation of cells, immune response, host-pathogen interactions and so on, it is also an essential part of the lipopolysaccharide receptor complex to transmit lipopolysaccharide induced signal to the cell. In addition, CD14+ is an part of the mechanism by which macrophages interact with apoptotic cells and engulf them. [28]. Based on the data obtained, we should say that hepatitis itself, without complication, does not lead to any significant change in these basal processes of immune system. Upward trend in CD14+ in the second group of patients may indicate the need to fight against foreign antigens released not so much by hepatitis C virus, as by the pathogens causing pneumonia: otherwise CD14+ should be also raised in the first group.

We used CD14+ & LBP as markers of immune responce against HCV-associated endotoxemia. Both LBP and CD14+ do not exclusively bind to lipopolysaccharide. Conversely, other antimicrobal peptides, independent of LBP and CD14+ are able to activate the immune system too. Since we did not find a significant increase in CD14 +, but LBP increased approximately 20 times, this makes us confident that this change in LBP is not associated with CD14 +.

CD16+ is the most well-researched membrane receptor implicated in triggering lysis mediated by natural killer cells. CD16+ is also known as FcγRIII receptor, which is found on the surface of macrophages, monocytes, neutrophils & natural killer cells [29]. CD16+ is a molecule of the immunoglobulin superfamily involved in antibody-dependent cellular cytotoxicity, which participate in cell signal transduction [30].Our studies have revealed, that in patients with viral hepatitis C and hepatitis C complicated by pneumonia, there is no significant change in this part of the regulation of the immune system. Therefore, we can conclude that this lineage of the immune defense also did not suffer from hepatitis C virus. In means, that the number of activated T-lymphocytes is reduced due to the decrease in CD25+ in hepatitis C (table 2). T-lymphocytes with receptors for interleukin-II, namely CD25 +, were reduced more than any other lymphocytes analyzed. These T-lymphocytes stimulate antibody production and cytotoxicity due to the interaction of foreign substances with specific antibodies. Indicator CD25+ characterizes lymphocytes proliferation state and reflects their ability to differentiate. CD25+ also characterizes the functional state of activated T-lymphocytes. We found a significant decline in CD25 + in patients with hepatitis C complicated by pneumonia, and to a lesser extent in patients with viral hepatitis without pneumonia: in both groups, the decrease in this indicator was obvious. A reduced amount of CD25+ indicates an immunological insufficiency of the cellular link of immunity, while overexpression of CD25+ could indicate increased activity of immune system. It is acknown that T lymphocytes are able to destroy targeted cells on direct contact; stimulated by the antigenic material presented by the macrophages, T cells produce lymphokines that signal other cells and thus trigger an immune response. Summarizing the data obtained, we can conclude that immune deficiency manifests itself precisely at this point of the response, which is associated with CD25-positive cells. It's obvious, that precisely CD25+ cells insufficiency opens the way for long-term viral activity in patients and their susceptibility to infection.

Some studies have reported that CD4 + / CD25 + levels increased in patients with chronic hepatitis C, but not in those who cleared the infection [6]. In our case this indicator changed from 1.58 to 2.49 and 3.62 in the I and the second groups, respectively. Ergo, we reaffirm that an increase in CD4+/CD25+ with HCV infection does open up the possibility of an exacerbation of the disease.

We found a tendency to an increase in the percentage of T-lymphocytes in the in the blood of patients with viral hepatitis C of group II, and a significant increase in T-lymphocytes of group I, but the percentage of B-lymphocytes significantly changed only in I group: it decreased. Since the immune response changes in the direction described above, these data may indicate a lower implication of antibodies, and more - of cytotoxic T-lymphocytes in hepatitis C. These findings may also indicate that destructive effect of HCV is manifested more in relation to the B-lymphocyte lineage.

The formation of immune complexes (CIC) is a physiological process which constitutes an essential part of normal immune defense mechanism. CIC production is generally followed by one or more secondary reactions. CIC, or antibodies associated with antigens that have penetrated the various body barriers, enable the body to neutralize microorganisms and clear from non-self-molecules. Otherwise microorganisms might multiply and form their inviolability zone, and their toxins induce damage mediated by specific ways. Inactivation and elimination of foreign invaders prevents their localization in the tissues [31; 32]. In group I, CIC was found increased 1.3 times, in the second - 1.6 times. In group II, a tendency towards a decrease in B-lymphocytes was found, and in group I - a significant decrease, but the antibodies secreted by activated B-lymphocytes circulate in the blood, attaching antigens, protecting the body and resulting in increase of CIC; hence the decrease in B-lymphocytes in these groups may be due toa drop in the number of lymphocytes, which are not responsible for the secretion of antibodies.

As it becomes clear after the interpretation of the test results, despite our multiple routine analyzes of classical indicators of hepatitis, nevertheless the most pronounced changes were found in the indicators reflecting the content of antimicrobal peptides, for example, defensin raised 7.7 times in first group versus control, and even 38 times – in the second group of patients. Defensins are active against viruses, bacteria &f ungi. Some α- and β-defensins are involved in signalling between the innate and adaptive immunity. Taking into account, that in the pathogenesis of hepatitis caused by HVC, the leading role belongs to endotoxins of gram-negative bacteria, especially their lipopolysaccharide endotoxins [33], one can easily get how important is to study the role of endotoxemia markers in the development of chronic hepatitis caused by HVC. Endotoxins, a part of which are represented by lipopolysaccharide agents, are the factors of Gram-negative bacteria pathogenicity, implicated in the development of Gram-negative infection complicatios, leading even to shock and death. Lipopolysaccharide endotoxin reacts with lipopolysaccharide-sensitive cells, which in turn produce endogenous mediators, for example tumour necrosis factor alpha. The toxic action of lipopolysaccharide and tumour necrosis factor alpha can be mediated via macrophages [34]. Actually, an increase in endotoxine levels found in our studies, reflects level of persistence of the infection.

The increase of defensins in our study reflects the effort of organism to eliminate toxins. During immune responce, CD14+, a cluster for LBS action, acts as a co-receptor for the detection of bacterial lipopolysaccharide. Additionally, it is known that CD14+ binds lipopolysaccharide only in the presence of LBP. Bacterial lipopolysaccharide is a main ligand for CD14+, expressed mainly by macrophages, CD14+May also recognizes other pathogen-associated molecular patterns.

By comparing the change in CD14+ (small increase) with changes in LBS & endotoxine (dramatically increase), we may conclude, that problem in patients of I & II groups was not so much in absence of LBP or defensin, as failure to synthesize significant number of clusters CD14+ to mediate action of LBP.

Lipopolysaccharide is accepted as the main toxic component of Gram-negative bacteria [35]. *In vivo,* a high concentration of LBP decreases the activity of bacterial lipopolysaccharide and can protect against septic shock caused by Gram-negative bacteria [36]. LBP is an acute phase protein secreted primarily by hepatocytes [37].The transcription of LBP gene is mainly stimulated by lipopolysaccharide of Gram-negative bacteria, IL-1 and IL-6. LBP can also bind to other lipopeptides, thus not being lipopolysaccharide exclusive; but free or membrane-bound lipopolysaccharide is the main ligand for LBP. Along with it, CD14+ might also interact with other pathogen associated molecular patterns, not being specific cluster of differentiation for the lipopolysaccharide –LBP complex only [34]. Since LBP is synthesized as hepatocytes response to infection, we were interested in the changing of concentration of LBP in order to reveal the activity of the inflammatory process in the liver. The levels of LBP correlated with the severity of the infectious process in this tissue, and the highest levels of this indicator was noted in the second group: compared with the control in this group, the levels of LBP increased 18.8 times (443.6 ± 29.6 mg / ml). For comparison, in group I the same indicator increased 5.9 times (138.7 ± 8.9 mg / ml). Thus, we argue that the more active is infectious process, the higher the LBP levels. On the one hand, an increase in the level of LBP is a marker of endotoxemia severity, but on the other hand, this may indicate an increase in the immune defense against endotoxins.

It is acknown that the natural killer T cells lose CD3 under the action of gamma-interferon, which is produced in response to viruses in the body. These cells belong to the natural killer cell line that are distinguished by the presence of CD56 and the absence of CD3 (CD56 +, CD3 -) [Malone D.F. et al., 2017]. In contrast to natural killer T cells, these cells usually express the surface markers CD16 (FcγRIII). In our research, CD 3+ & CD16 changed unsignificantly. Romee R. et al. [38] disclosed, that activated natural killer cells lose CD16 (FcRγIII) through a metalloprotease-17, which is why inhibition of ADAM17 metalloproteinase stimulates CD16 mediated natural killer function due to preserving CD16 on the NK cell surface; that in turn enhances antibody-dependent reactions of cellular cytotoxicity to cleanse the body of foreign presenters. CD16 on natural killer cells induce genes for transcription of IL-2-R (CD25) and inflammatory cytokines such as gamma-interferon and tumor necrose factor. Then cytokine activation and target cell stimulation leads to marked decrease in CD16 expression. Romee et al. state that the activation of natural killer cells by binding CD16 to antibodies results in a loss of CD16, and this process correlates with increase of gamma-interferon. Expressed by natural killer cells metalloprotease-17 in turn suppresses the expression of CD16, which leads to increased production of gamma-interferon, especially when it has been triggered through CD16. All this indicates the need to involve mainly the interferon link in the process of body self-defense in viral hepatitis C. These data also indicate the presence of reciprocity in the mechanism of immune defense; thus, the enhancement of the interferon link correlates with the softening of the cellular link of the immune defense. Apparently, this is the main reason for the absence of a significant increase in the CD16 titer in our studies: it is more likely to speak of a downward trend than an increase in this line of lymphocytes.

5. Conclusion

We assume that CD^{25+} and endotoxin are more informative indicators for diagnosing HCV than routine tests. LBP & defensin may be indicators of immune response in HCV infection as well. Finally, this research helps to develop laboratory methodology towards the introduction of sensitive diagnostic assays.

Compliance with ethical standards

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Authors' Contributions

We, the authors of this manuscript declare, that the article is an honest work, and agree with the above-listed contributions of each author to the result of the work.

- **Mahira Amirova:** guarantor: writing- original draft preparation, conceptualization.
- **Gulnara Azizova:** data acquisition, review.
- **Arzu Dadasheva:** design, data acquisition, literature search.
- **Ellada Huseynova:** experimental studies.
- **Gulnara Vahabova:** data analysis.

Disclosure of conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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