Proinflammatory Cytokines IL-2, IL-6 and TNF Alpha as Immunoserologic Indicators of Chronic Viral Hepatitis B and C in Children

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Abstract The role of proinflammatory cytokines IL-2, IL-6, and TNF- α in the pathogenesis of chronic hepatitis B and C infection was studied in 49 children ages 3 to 18 years. There has been concluded that high IL-2 and low IL-6 serum levels related to hepatic inflammatory activity did not depend on viremia levels. Slow progression of the disease was associated with an insignificant increase of IL-6 and normal TNF- α serum levels. The study results show the importance of proinflammatory cytokines as diagnostic and prognostic indicators in the pathogenesis of chronic hepatitis B and C in children.

Keywords Cytokines, Children, Chronic Hepatitis B and C Infection

C viral infection course in children have been the subject of multiple scientific studies for a long time [2, 3]. In addition, factors like infection pathway, age, antigenic properties of the pathogen and its adaptive capacity [2, 3] have played a major role in the occurrence of chronic hepatitis B (CHBV) and C (CHCV) viruses. Active replication of HBV starts only 4-5 weeks after the penetration of the causative agent into liver cells [3, 7]. Evolution of chronic hepatitis B in children is characterized by the predominance of the immunoactive phase [1].

An important characteristic of HBV is the ability to integrate into the human genome, causing a change in the sequence of nucleotides and a violation of antigen expression with the formation of HBsAg-negative forms of HBV [3, 5]. A significant role in the pathogenesis of HBV infection is the ability of the causative agent to cause mutations in the S-region or pre-S-region, thereby modifying its antigenicity and suppressing the synthesis of anti-HBs, creating conditions for itself to escape from the immune control of the organism [2, 3].

Compared to chronic HBV infection, the main role in the pathogenesis of chronic HCV (CHCV) infection belongs to the direct cytopathic effect of HCV and direct replication of the virus to billions of copies per day immediately after its penetration into the hepatocyte [2, 3]. There is an opinion that, in chronic HCV infection, the major role in the elimination of the virus from the body is played by cellular immune responsiveness between antigen-specific T-helper 1 (CD4 +) and cytotoxic T-lymphocytes (CD8 +) [2, 3]. The body protective immune response has an indirect role in the damage of infected hepatocytes of HCV and is provided by the action of cytotoxic T-lymphocytes and cross autoimmune reactions [2, 4]. The variability of HCV and its low immunogenicity are of major importance for a long-term persistent replication [2, 3]. By changing continuously its antigenic structure, HCV escapes from the body's immune

1. Introduction

Pathogenetic mechanisms and factors that lead to chronic hepatitis B and C viral infections in children, including the perinatal ones, as well as the role of proinflammatory cytokines have not been sufficiently studied yet. The success of elimination and resolution of hepatitis B and C viral infection depends on the age and the immune status of the patient. Most chronic HBV and HCV perinatal infections commonly occur by both vertical (from mother to child) and horizontal transmission, as well as in immunocompromised patients. The immune determinants of a successful HBV clearance haven't been entirely understood, whereas both cellular and humoral immune responses are important. Nevertheless, liver inflammation and disease are also believed to be largely immune-mediated. Cytokines are important mediators between specific and nonspecific, humoral and cellular immunity, providing a protective response of the body against the inflammatory process [1, 4, 8]. Pathogenetic mechanisms and factors contributing to the chronic B and

control, reproducing itself intensively [2, 3]. This variability of HBV determines the development of subclinical, erased and difficult to diagnose "occult" forms of chronic HBV rarely seen in children.

Proinflammatory cytokines-interleukin-2 (IL-2), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α) play an important role in both detection of HBV or HCV and suppression of its replication, which contributes to the regulation of the equilibrium of subpopulations of T-lymphocytes, their further activation, differentiation of effector cells and the synthesis of anti-inflammatory cytokines [4,7]. Proinflammatory cytokines IL-2, IL-6, TNF- α are activated from the onset of HBV or HCV replication and the increase of viremia titer [2, 3]. According to their functional activity, cytokines are divided into pro-inflammatory (IL-1 β , IL-2, IL-6, TNF- α , INF- γ) and anti-inflammatory (IL-4, IL-10) [7, 8]. T-helper cells via cytokines recognize the pathogen and stimulate activation of T-killer lymphocytes to fight off the infected cells, whereas the T-suppressor lymphocytes help suppresses this process. [9].

The main synthesis of IL-2 is carried out by T-helper lymphocytes, cytotoxic T-cells up to 20%, and can be stimulated by other biologically active substances like IL-1, IL-6, TNF [8]. The proinflammatory cytokine IL-2 is the main triggering pathogenetic factor of a specific and non-specific immune response to pathogenic antigens, which promotes the activation of IL-2 receptors located on T-lymphocytes, influences maturation of B-lymphocytes, stimulates the formation of acute phase proteins in hepatocytes, neutrophils NK and makes connection between the immune, nervous and endocrine systems [7, 8].

IL-6 belongs to a group of inflammatory cytokines synthesized by macrophages, T and B-lymphocytes, and which provides proliferation of thymus cells, B-lymphocytes, cytotoxic lymphocyte precursors, granulocytes and macrophages, as well as hepatocyte production of acute inflammatory proteins [7, 8]. TNF- α is produced by macrophages, mast cells and T- and B-lymphocytes and refers to lymphotoxins that acts as the penetration gate of the pathogen that induces the synthesis of acute inflammation and apoptosis proteins [2, 3, 8].

IL-4, IL-13, IL-10 cytokines help in regulation and control of the level of proinflammatory cytokines in blood [7, 8]. T-helper 1 (Th1) lymphocytes have been shown to promote activation of cytotoxic T suppressor (CD8 +) by T-helper 1 (Th1) and enhance the synthesis of interferon- γ (INF- γ), IL-2, TNF- α) [2, 3, 4]. Activation of T-helper 2 (Th2) lymphocytes stimulates the humoral immune link, promoting the maturation of B lymphocytes and the synthesis of anti-inflammatory cytokines [3, 8]. Activation of the immune response system by Th1-type promotes the removal of the virus from the body, whilst Th2-type promotes the persistence of infection [2, 8]. Recent studies have shown the presence of T helper (Th1

and Th2) CD4 + lymphocyte deficiency in the blood of patients with CHCV infection [2, 3]. This can lead to a breakdown in the synthesis of proinflammatory and anti-inflammatory cytokines, their inactivation and cytokine balance, as well as formation of chronic inflammation [7, 8].

Objectives: To study the serum level of proinflammatory cytokines IL-2, IL-6, and TNF- α in children with chronic HBV and HCV infections, depending on both the activity level of chronic viral inflammatory process in liver and viremia level.

2. Materials and Methods

2.1. Study Group and Methodology

The study was conducted on 181 children with chronic viral hepatitis B (in 107 cases) and C (in 84 cases), ages 3 to 18 years [Table 1]. In order to determine the particularities of the cellular immunity in 50 children with CHBV (in 25 cases) and CHCV (in 25 cases), we proposed to study the cellular immune status of CD3 T lymphocytes and their subpopulations before the study and after undergoing the antiviral treatment [Table 2]. Cellular immunity has been studied in 49 patients by using monoclonal reactants, whereas the levels of pro-inflammatory cytokine IL-2, IL-6 and TNF- α have been studied in 49 children with chronic HBV (in 30 patients) and HCV (in 19 patients) [Table 3]. The control group included 21 "somatically healthy" children aged between 4-18 years.

The diagnosis of chronic HBV and HCV was defined in accordance with international and national recommendations [9, 10]. The methods used in survey data collection are the anamnesis, examination of the patient, investigation of virological markers as HVB, HVC, ADN HVB, ARN HVC and their genotype, ARN VHD (PCR by Real Time).

According to clinical indications, magnetic resonance abdominal tomography (MRT) was performed. No investigation on HBV genotype was conducted. The degree of liver fibrosis was examined in children over 5 years with a weight of more than 20 kg via elastography (FibroScan 502, device Ecosens, France). A liver biopsy was carried out by the author, via Menghini needle puncture under general anesthesia, and followed by histological examination of the biopsy specimen.

The detection of IL-2, IL-6, TNF- α was performed in the clinical immunology laboratory via the immunoenzyme test system (manufactured by «Vector-Best», Russia). The participation of the patient in the research was confirmed by the written consent of the parents. The scientific project was approved on September 14, 2015 by the Ethics Research Committee of the "Nicolae Testemitanu" State University of Medicine and Pharmacy from the Republic of Moldova.

Table 1. Baseline characteristics of patients with CHBV and CHCV infection.

Characteristics	CHVB n=107 (59,1%)	CHVC n=74 (40,9%)	total, n=181	p
Mean age, years	10,7±0,5	11,4±0,5	11±5,2	>0,05
Mean age diagnostic, years	6,3±5,3	5,4±0,5	3,5±0,4	<0,05
Sex: boys, n (%)	64 (59,8)	46 (62)	110 (60,8)	
girls, n (%)	43 (40,2)	28 (38)	67 (39,2)	>0,05
urban, n (%)	33 (31)	34 (46)	67 (37)	<0,05
rural, n (%)	74 (69)	40 (54)	114 (63)	<0,05
BMI*, kg/m ²	18,3±2,5	18,7±2,9	18,5±2,7	
Mean Bi total*, mcmol/l	15,8±1,1	20,1±3,2	17,5±19,8	>0,05
Mean ALT*, ui/l	66±5,6	73±9,8	69±5,2	>0,05
Mean AST*, ui/l	55±6,3	59±6,5	59±4,6	>0,05
Mean viremia*, ui/ml	1 914 201 586±	2 871 671±	9 170 372 876±	>0,05
	1 272 910 855	1 137 741 (67)	731 875 432	>0,05
AgHBe positive, n (%)	70 (65,4%)	-	-	
Genotype VHC, abs (1/2/3a)		66/2/6	-	<0,001
Mean elasticity*, kpa (n)	5,8±0,4 (24)	5,4±0,2 (47)	5,5±0,2 (71)	>0,05
Fibrosis degree, n (%), including:				
F0-F1	27 (23%)	33 (45%)	60 (33)	<0,05
F1-F2	17 (63%)	27 (82%)	44 (73)	
F2-F3	4	6	10 (6)	
F3-F4	4	0	4 (2)	
	2	0	2 (1)	
IAH (biopsia)*, (n)				
INF alfa 2b peg (mono, 24 weeks;	8±1,8 (19)	4±2,1(6)	7,3±1,5 (25)	>0,05
combined and ribavirin 24-48 weks), n	48 (46)	48 (66)	96 (53)	
(%), inclusively:				
SVR, n (%)	15 (31)	29 (60)	44 (45,8)	<0,001
PVR, n (%)	16 (33)	11 (23)	27 (28)	<0,001
NR	17 (35)	8 (17)	25 (26)	<0,001

Note: * ± indicate standard deviation, BMI - body mass index Kettle, ALT – alaninaminotransferase, AST – aspartataminotransferaze, Bi – total bilirubin, HAI- histological activity index, SVR- sustained virologic responder, PVR- partial virologic response, NR – non responder.

The statistical analysis was carried out via StatSoft Statistica version 6.0 ru., which identified t-Student criterion, the exact Fisher test and the chi-square test. Cases with $p \leq 0.05$ were determined to be statistically significant.

Table 2. Characteristics of cellular immunity in CHBV and CHCV –infected children, % (abs)

Characteristics	Control group (n=22)		CHBV (nr=107)		CHCV (nr=74)		p _{1,2} ; p _{1,3} ; p _{2,3}
	1		2		3		
	nr	M±m	nr	M±m	nr	M±m	
CD3,T total lymphocyte, % CD3, abs	22	66±1,4 1,8±0,2	25	52±2,8 1,6±0,3	25	60±1,3 1,6±0,2	<0,001,<0,01,<0,01 >0,05,>0,05,>0,05
CD19, B total lymphocyte, % CD19, abs	22	18±1,0 0,58±0,1	25	13,4±1,7 0,33±0,1	25	12,3±1,2 0,36±0,1	<0,05 <0,001 >0,05 <0,05 <0,05 >0,05
CD4, T helper lymphocyte,% CD4 abs	22	39±1,8 1,1±0,2	25	36±1,8 1±0,2	25	36±1,3 0,9±0,1	>0,05 >0,05 >0,05 >0,05 >0,05 >0,05
CD8,T suppresor lymphocyte ,% CD8, abs	22	27±1,1 0,8±0,1	25	18±2,3 0,48±0,1	25	23±1,0 0,67±0,1	<0,01 <0,05 <0,05 <0,05 >0,05 >0,05
CD4/CD8	22	1,43±0,1	25	3,06±0,6	25	2,05±0,4	<0,05 >0,05 >0,05
(CD4+CD8)/CD3	22	0,97±0,02	22	1,05±0,04	22	0,97±0,02	>0,05 >0,05 >0,05
CD5,T mature lymphocite, % CD5 abs	22	61±0,97 1,7±0,1	22	47±3,4 1,2±0,1	22	52±1,9 1,7±0,2	<0,001<0,001>0,05 <0,05 >0,05 >0,05
CD16, natural killer (NK), % CD16 (NK, abs)	22	12±1,2 0,33±0,04	15	14,7±1,1 0,32±0,03	15	18±0,9 0,44±0,1	>0,05 <0,001<0,05 >0,05 >0,05 >0,05
CD HLA DR, T lymphocyte activation/ B lymphocyte, % CD HLA DR, abs	22	29,9±1,98 0,9±0,1	17	21,6±2,5 0,5±0,1	17	20±1,6 0,6±0,1	<0,05 <0,001>0,05 <0,001 <0,01 >0,05

Investigations of the cellular immune status in children with CHBV and CHCV revealed the presence of total T-cell lymphocyte imbalance, expressed by a decrease of CD3, CD19, CD4+, CD8+, CD5, CD HLA DR and a disturbance of the immune balance; an increase in the CD4 +/CD8 + immunoregulatory index; an increase in the percentage of CD16 natural killer cells; the presence of the „double positive” phenotype (CD4 +, CD8 +) > CD3 in 36% of children with CHBV and in 41% of CHCV patients [Table 2]. These confirmed the involvement of autoimmune self-defense mechanisms under „chronic infectious stress” at viral antigen stimulation against the background of T -lymphocyte immune deficiency.

3. Results and Discussions

The average age of children with CHBV and CHCV

was 11 years, predominantly boys. In 18% of children with HBV, the disease was associated with delta infection. Among children with chronic HBV, a pronounced fibrosis of the liver F3 was detected in 3 (21%), F2 in 2 (15%), and F0-F1 in 9 cases (64%) respectively. Genotype 1c prevailed in 96% of patients with CHCV and only one child presented genotype 3a. CHCV was characterized by minimal fibrosis F0-F1 - in 9 patients (64%), F2 in 5 patients (36%) and lack of F3.

CHBV and CHCV were characterized by an active inflammatory process in 57% of patients. The ALT enzyme was more than 1.5 times greater in 13 children, 3 times higher in 14 children and only in 5 cases the values exceeded by 4-10 times the permissible standards. 50% of HBV-infected children were characterized by immune-active phase with HBV DNA ranging between 2000 - <109 / ml and minimal fibrosis".

Table 3. Serum level of cytokines in CHBV and CHCV-infected children

Cytokines	Control group (n=21)		CHBV (nr=30)		CHCV (nr=19)		p _{1,2} , p _{1,3} , p _{2,3}
	p ₁		p ₂		p ₃		
	nr patients	M±m	nr patients	M±m	nr patients	M±m	
IL-2, pg/ml	21	1,5±0,3	30	108±47	19	113±34	<0,05 <0,05 >0,05
IL-6, pg/ml	21	4,5±0,4	30	41±22	19	29±14	>0,05 >0,05 >0,05
TNF-α, pg/l	21	4±0,5	30	7,6±0,7	19	7±0,5	>0,05 >0,05 >0,05

Note: M ± m – mean and statistical deviation; p– comparison between groups of patients; statistical tests: t-Student; Fisher criterion.

Immunotolerant phase with a high level of viremia and HBV DNA range of 10^9 - 10^{12} ui / ml among the examined patients was not detected. In 6 children with HBV, a delta infection with a high level of viremia and HDV RNA range of $1,3 \times 10^8$ – $1,4 \times 10^9$ ui/ ml was diagnosed. The development of CHVC was characterized by low viral replication in 61% with a HCV RNA range of < 600 000 ui / ml and absence or minimal liver fibrosis.

Analysis of the serum level of proinflammatory cytokines revealed a significant increase in the level of IL-2 in blood, compared to the control group ($p_{1,3} < 0,05$, $p_{2,3} < 0,05$) in CHBV and CHCV –contaminated children (Table 3).

The level of IL-6 was higher among children with CHBV infection, compared to children with CHCV ($p_{1,3} < 0,05$). Given that IL-6 promotes the differentiation of T-lymphocytes towards Th2 (CD4 +), which suppresses the immune response, the relatively low concentration of IL-6 in CHVC compared to CHVB has been associated with a minimal activity level of inflammatory process in the liver.

A slight but significant increase in the serum level of IL-6 and minimal fibrosis in the examined HCV patients is probably due to a weak immunological response and low HCV replication due to insufficient differentiation of T-lymphocytes. The study of the serum level of TNF-α in children with CHBV and CHCV did not reveal any significant changes (Table 3).

TNF-α is known as a local-action proinflammatory cytokine, which activates those factors responsible for the acute inflammatory process induced via blood, and correlates with the disease severity and a good immune response.

A comparative analysis of the concentration of proinflammatory cytokines IL-2, IL-6 and TNF-α was performed based on the degree of activity of the inflammatory process of CHBV and CHCV infection and the level of viremia.

A direct and reliable relationship between the IL-2 indices in the blood and the activity of the inflammatory process was revealed [Figure 1]: the higher the cytotoxicity, the higher the level of IL-2 in CHBV and CHCV-infected children ($p_{1,2,3} < 0,05$). The TNF-α values in blood of the examined patients were within the permissible limits, whereas the concentration of IL-6 in blood of CHBV and CHCV-infected children was slightly increased and did not depend on the degree of activity of the inflammatory process ($p_{1,2,3} > 0,05$).

It is necessary to mention that in CHBV-infected children, the serum values of IL-6 and TNF-α show no diagnostic significance since the disease develops into the immune-active phase with low viraemia over the years. Therefore, there has been detected a presence of imbalance in the synthesis of proinflammatory mediators followed by a secondary immunodeficiency within studied children.

Figure 1. Blood serum level of proinflammatory cytokines IL-2, IL-6 (in pg/ml), TNF-α (pg/l) in CHBV and CHCV – infected children

An analysis on relationship between the blood serum level of IL-2, IL-6 and TNF-α and the level of HBV and HCV replication has been carried out. The concentration of IL-2 and IL-6 in blood was significantly increased in CHBV-infected patients with DNA HBV within $> 2000 - 10^8$ ui/ml, whereas for those with CHBV infection the DNA less than 2000 ui/ml - higher values ($p > 0,05$).

Among children with CHCV and ARN HCV $< 600\,000$ ui/ml, the level of IL-2 and IL-6 in blood was significantly higher than in viremia more than 600 000 ui/ml ($p > 0,05$).

The serum level of TNF-α in CHBV and CHCV -infected children did not reveal dependence on the level of viremia. Taking into account that 50% of children with HBV infection manifested an immunoactive phase, and 61% of HCV cases presented low HCV replication, the lack of a reliable relationship between the serum levels of IL-6 and TNF-α and the level of viremia might be due to the interaction of complex regulatory mechanisms which activate various factors and inhibit cytokine production. The slow maturation of cellular and humoral factors of innate and adaptive immunity in children reaches its perfection by 6-7 years [2, 8]. HBV or HCV infection in younger children leads to the formation of partial and insufficient immune responses specific to the introduction of the pathogen, which creates conditions for its prolonged persistence in the body.

4. Conclusions

Chronic Hepatitis B and C viral infections in children occupy an important share in the etiological structure of chronic hepatitis in children overall, whereas CHBV is

predominantly affecting adolescents, and represents a life-threatening issue for public health care services. The primary source of contamination of children with HBV and HCV infections is their HBsAg positive mothers or CHBV and CHCV during the viral active phase.

The basis of pathogenetic mechanisms of both inflammatory processes and fibrogenesis in viral hepatitis B and C in children are cellular immune reactions that result from complicated interaction between HBV or HCV and the infected child's organism.

The pro-inflammatory cytokines IL-2, IL-6 and TNF-α have an important diagnostic and prognostic significance in the pathogenesis of CHBV and CHCV in children. The high serum level of IL-2 in children with CHBV and CHCV infections significantly correlates with the level of activity of the chronic inflammatory process in the liver and does not depend on the level of viremia. IL-2 in children with CHBV and CHCV is a prognostic predictor of the persistence of inflammatory activity in the liver on the background of a secondary immunodeficiency.

A slight increase in the serum level of IL-6 and a normal level of TNF-α in CHBV-infected children in the immune phase and CHCV infection with a low degree of viremia confirm the inadequacy of both synthesis and protective immune responses, as well as the violation of regulatory mechanisms in the system of mediators of chronic viral inflammation.

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Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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