CD81 and CD5$^+$ B lymphocytes and HCV: Trojan horse for a cruel killer.

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Abstract: Background: HCV is hepatotrophic and lymphotrophic virus which may partly explain that Chronic hepatitis C virus infection is frequently associated with extra hepatic autoimmune phenomena. Receptors for HCV on B cells include the low density lipoprotein receptor and CD 81 which is a component of the complement receptor 2. CD5$^+$ B cells is reported to be expanded in chronic HCV infection. **Aim of the Work:** The aim of this work is to study the possible role of peripheral B-cell CD81 and CD5 in the development of HCV-related autoimmunity and their response to interferon therapy . **Materials & Methods:** This study was conducted on 45 subjects divided into two groups; **Patient group** : included 30 chronic HCV Egyptian patients , 26 males and 4 femaless with mean age 50±2 years, 17 patients were under treatment with interferon alpha plus ribavirin and 13 patients were not under therapy and **Control group**; included 15 normal subjects , 10 males and 5 females with mean age 46±14 years . **Results:** ALT and AST were statistically significantly higher in the patients group compared to the control group**;** ALT [80.9±45.3 vs. 7.1±2.2…], AST [60.2±51.8 vs. 6.4±3.1] and p <0.001 for both comparisons. The level of B-cells expressing the CD5 antigen in the peripheral blood of patients group was significantly increased over that of control group [23.2±7.2 % vs. 7.15±5.5 % and, p < 0.001]. In addition, the mean fluorescence intensity of CD81 expression was significantly higher in patients group than in control group [150±15 vs. 85±13. And, p < 0.001].CD5 percent was significantly positively correlated with the viral load in patients with significant fibrosis ie those planned for treatment [r = + 0.48457 and p < 0.05] , but after 6 month of therapy the correlation was statistically non-significant. however, CD81 was statistically non-significantly correlated with the viral load in that group of patients before starting treatment , but showed statistical significant positive correlation with the viral load after 6 month of therapy [r= 0.55539 and p < 0.05].ANA, antismooth muscle antibody, rheumatoid factor, and cryoglobulins were significantly more prevalent in HCV patients than healthy controls [30% vs. 6.7%, 60% vs. 6.7%, 60% vs. 13.3% and 40% vs. 0%, respectively and, p < 0.05 for all comparisons]. The expansion of CD5+B-celIs was found to be significantly associated [p < 0.05 for all correlations]with the production of RF, ANA, LKM, ASMA and MC . **Conclusion:** overexpression of CD81 and the expansion of CD5$^+$ peripheral B-cells in HCV infected patients may possibly play a role in the development of HCV associated autoimmunity and that IFN- ribavirin treatment down regulates cell surface CD81.

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1. Introduction

HCV may directly stimulate and infect B lymphocytes. Receptors for HCV on B cells include the low density lipoprotein receptor and CD 81 which is a component of the complement receptor 2. CD81 is a 26-KDa surface protein. It is composed of four trans membrane and two extracellular domains. Its functional effects include adhesion, activation, proliferation and differentiation of B lymphocytes, T lymphocytes and other cells. CD81 is a part of complex with CD21, CD 19 and leu 13. This complex reduces the threshold of B-cell activation via the B-cell receptor. CD 81 binds HCV through interaction with its envelope glycoprotein E2. Binding of HCV E2 glycoproteins mediates internalization of 30% of CD81 molecules after 12 hours. CD81 is also involved in HCV assembly and secretion. Antibodies neutralizing the E2 glycoprotein may block the binding of HCV to CD81 and have been shown to correlate with protection against HCV infections in chimpanzees [1].

CD5$^+$ B cells is reported to be expanded in chronic HCV infection. These cells are characterized by the production of low –affinity immunoglobulin M [IgM] with rheumatoid factor activity. Also, they have been identified in the hepatic lymphoid follicles of HCV-infected patients where they may have a protective role decreasing disease progression and they, in addition, Produce both monoclonal and polyclonal IgM **[2].**

HCV is hepatotrophic and lymphotrophic virus which may partly explain that Chronic hepatitis C virus infection is frequently associated with extra hepatic autoimmune phenomena and the presence of autoantibodies such as Antinuclear antibody[ANA], antismooth muscle antibody[ASMA], liver kidney

microsomal antibodies [LKM Ab], mixed cryoglobulinaemia [MC] which is a benign lymphoproliferative disorder, autoimmune thyroiditis and Sjogren's like syndrome [3] . IFN-a is a cytokine with potent effects of gene transcription and translation, leading to a variety of changes in cellular proteins and cell surface receptors. It has a role in immunomodulation against HCV [4] .

Aim of the Work:

The aim of this work is to study the possible role of peripheral B-cell CD81 and CD5 in the development of HCV-related autoimmunity and their response to interferon therapy .

2. Materials & Methods:

This study was conducted on 45 subjects divided into two groups ; **Patient group** : included 30 chronic HCV Egyptian patients , 26 males and 4 femaless with mean age 50±2 years ,17 patients were under treatment with interferon alpha plus ribavirin and 13 patients were not under therapy and **Control group**; included 15 normal subjects , 10 males and 5 females with mean age 46±14 years (Table I).

All the subjects were subjected to the following investigations: I. Complete blood count 2. Liver function tests: ALT, AST, GGT, PT, Total Bilirubin, serum albumin 3. HCV antibody by ELISA confirmed by RIBA IV test. 4. Peripheral B-cells CD5 expression and mean fluorescent intensity (MFI) of surface CD81. 5. Quantitative PCR were done for all HCV patients (n=30).6. For the 17 patients under treatment, quantitative PCR were repeated 1, 3 and 4 months after starting therapy. 7. Surface CD81 was repeated 1, 3 and 4 months after treatment for the patients under therapy 8. Autoimmune markers (ANA, ASMA, LKM, Anticardiolipin antibody, cryoglobulins) were done for all subjects under study.

Laboratory methods: I. Peripheral B-cells CD5 expression and MFI of CD81 was done using flow cytometry; After separation of monoclonal layer, using Ficoll hypaque density gradient, 100°L of monoclonal cell layer was added to 100^{o} L monoclonal antibody, incubated for 15 minutes in refrigerator, washed with phosphate buffered saline and read as percent expression[5] [(5)].B-cells were immunostained directly (one step) with PE and FITC conjugated monoclonal antibodies for CD81 and CD5 (Becton Dickinson, Oxford VR).Flow cytometry was carried out with a fluorescent activated cell scan (FACS). The total population of viable cells was gated according to their typical forward and right angle scatter. The fluorescence of cells treated with fluorescent isotype Mo Ab was evaluated in each experiment to determine the level of background fluorescence of negative cells.CD5 B-cells were expressed as a percentage of CD81 cells, the percentage and mean fluorescence intensity (MFI) of stained cells were determined according only to the positive cells. .MFI of CD81 was expressed as optical density units (OD) (Fig. 1). 2. PCR quantitative was done using the automated Cobas Amplicor HCV monitor assay, V. 2.0 (Roche Diagnostic, Switzerland). The assay is based upon reverse transcription and amplification of the HCV target using primers from the 5' non-coding region in the presence of internal quantitation standard (IQS) that is co-amplified with the target and competes for the primer, since they share the same primer region, each sample is quantified in relation to its IQS. RNA extraction and calculation of the number of HCV copies per ml were performed according to the manufacturer's protocol.3. Autoantibodies: was done using indirect fluorescence (FlourokitTM Diasorin, USA). Indirect irnmunomicroscopy for the screening and titration of circulating serum autoantibodies, rat kidney, rat stomach and liver cryostat secretions[6] [(10)].4. Cryoglobulins [7] [(12)]: Blood was collected and kept at 37°C for 30 min. prior to separation, serum separated from the clot by centrifuging the clot for 10 minutes at 2,500 rpm, a drop of sodium azide (0-1 g/L) is added to prevent bacterial overgrowth. Consequently, serum samples are observed at 40°C 7 days after collection. Positive sera are sundown in a refrigerate (4°C), centrifuge at 3.000 rpm for 15 min. The sample is suspended in warm saline I/JO to 1 volume of the initial serum) and incubated at 37°C for 1 hour. Positive precipitate should dissolve completely.5. RF was done by AVITEX-RF Omega Diagnostics.

3. Results:

ALT and AST were statistically significantly higher in the patients group compared to the control group**;** ALT [80.9±45.3 vs. 7.1±2.2], AST [60.2±51.8 vs. 6.4±3.1.] and, p <0.001 for both comparisons. The level of B-cells expressing the CD5 antigen in the peripheral blood of patients group was significantly increased over that of control group [23.2±7.2 % vs. 7.15±5.5 % and, $p < 0.001$]. In addition, the mean fluorescence intensity of CD81 expression was significantly higher in patients group than in control group [150±15 vs. 85±13 and $p < 0.001$] (Table 1).

CD5 percent was significantly positively correlated with the viral load in patients with significant fibrosis ie those planned for treatment [r = + 0.48457 and $p < 0.05$], but after 6 months of therapy the correlation was statistically non-significant. however, CD81 was statistically non-significantly correlated with the viral load in that group of patients before starting treatment, but showed statistical significant positive correlation with the viral load after 6 months of therapy [r= 0.55539 and $p < 0.05$].[Tables 2,3].

ANA, antismooth muscle antibody, rheumatoid factor, and cryoglobulins were significantly more prevalent in HCV patients than healthy controls [30% vs. 6.7%, 60% vs. 6.7%, 60% vs. 13.3% and 40% vs. 0%, respectively and p<0.05 for all comparisons]. [Table 4].

The expansion of CD5+B-cells was found to be significantly [$p < 0.05$ for all correlations]associated with the production of RF, ANA, LKM, ASMA and MC .

Table (1): Descriptive data of patients (n=30) and controls (n=15):

	Patients (n=30)	Controls (n=15)	*P*	
Age	50±12	46+14	>0.05	NS
Sex (M/F)	26/4	10/5	>0.05	NS
ALT (U/L)	80.9±45.3	7.1±2.2	<0.001	HS
AST (U/L)	60.2±51.8	6.4±3.1	<0.001	HS
$CD5^+$%	23.2±7.2	7.15±5.5	<0.001	HS
CD81 MFI	150±15	85±13	<0.001	HS

Table (2): Association between viral load and CD5 and CD81:

Group		HCV-RNA & $CD5^+$	HCV-RNA & CD81
All patients (n=30)	r	-0.44001	-0.49017
	Sig.	-S	-S
Pretreatment patients (n=17)	r	+0.48457	-0.45447
	Sig.	+S	-S
After 1 month (n=17)	r	+0.44886	-0.59268
	Sig.	-S	-S
After 3 months (n=1 7)	r	-0.48505	+0.4076
	Sig.	-S	+S
After 6 months (n=17)	r	-0.54757	+0.55539
	Sig.	-S	+S

Table (3): HCV-RNA viral load, CD5+ percentage and MFI of CD81 in patient groups:

Groups	HCV-RNA viral load	$CD5^+$ B-cells	MFI Of CD81
All HCV patients (n=30)	$522x10^3$± 470x1 O^3	23.2±7.2%	150+15
Patients not under therapy (n=13)	502x1 0^3± 322x1 O^3	21.3±5.8	143±17
Pretreament patients under therapy (n=17)	542x1 0^3± 444±10^3	25.1+6.3%	157±12
After one month (n=17)	317x10^3± 219±10^3	19.8±5.5%	130+20
After 3 months (n=17)	120x10^3± 11 2+1 0^3	15.6±3.9%	120±17
After 6 months (n=17)	65x1 0^3± 81±10^3	11.5±4.3%	100+25

Table (4): Autoantibodies in patients (n=30) and controls (n=15):

	Patients	Controls	*P*	Sig.
	(n=30)	(n=15)		
ANA	9 (30%)	1 (6.7%)	O.05	S
LKM	3(1%)	0 (0%)	>0.05	NS
ASMA	18 (60%)	1 (6.7%)	<0.001	HS
RF	18 (60%)	2 (13.3%)	<0.01	HS
Cryoglobulins	12 (40%)	0 (0%)	<0.01	HS

4. Discussion:

Hepatitis C virus (HCV) infection is one of the major causes of chronic hepatitis with frequent progression to cirrhosis and an elevated risk for the development of hepatocellular carcinoma Combination therapy with interferon alfa (IFN-a) and ribavirin leads to apparent eradication of HCV in 38% to 43% of treated patients [8].

The mechanism by which HCV enters target cells is still unknown. Recently, it was postulated that CD81 molecule binds HCV through interaction with the E2 glycoprotein. Compared with healthy subjects peripheral blood lymphocytes (PEL) from HCV infected patients showed higher CD81 MFI. However, IFN-ά and ribavirin combination therapy showed down regulation of CD81expression on PEL. This goes with agreement with **Rronenberger *et al.*** [9].

Higher levels of CD81 may confer a selective advantage for HCV either by modulation of the immune response or by increasing attachment of viral particles. Anticipating that CD81 may directly be involved in HCV life cycle, higher levels of CD81 could contribute to higher rates of de novo infection and secretion of HCV [9]. CD81 expression declined during therapy in patients with HCV-infection. This effect may be directly caused by IFN-ά or indirectly caused by viral decline [10] .In **Kronenbergetz *et al.,*** study; lower levels of cell surface-associated CD81 were associated with HCVgenotype-3 and the initial decline of HCV RNA after initiation of combination therapy [4]. In this study, the prevalent genotype is HCV-4 which is the common genotype among Egyptians.

Chronic HCV infection is associated with extrahepatic manifestations including autoimmune phenomena, benign expansion of B-cells and non-Hodgkin's lymphoma which suggest B-cell activation and proliferation. However, the exact mechanism linking HCV and autoimmunity is unknown [11]. In this study, we demonstrated that peripheral B-cell from patients with chronic HCV infection overexpress both CD5 and CD81 compared with normal controls. This increase correlated with the levels of HCV RNA and was also found to be significantly associated with the presence of autoimmune markers. **Zuckerman *et al.,*** also found that CD5+B which are capable of producing natural antibodies with auto reactive specificities are likely to be important in the development of HCV associated autoimmunity [11]. **Ivanovski *et al.,*** supports the role of chronic antigenic stimulation in inducing B-cell clonality in HCV-infected patients. B-cell proliferation in HCV-infected patients is probably enhanced by HCV-specific properties, including the ability of HCV-proteins to bind to CD81 on B-cell surface and to influence intracellular regulatory function following viral entry into B-cells [12].

Clifford and Colleagues observed a high prevalence of autoimmune markers in patients with chronic HCV. They found that these patients were 66% positive for ASM A, 14% positive for ANA but only 2% were positive for LKM. Compared to this study, we found 30% ANA positive, 1 % LKM, 18% ASMA, 60% RF and 40% MC positive[13] .**Fornasieri *et al.,*** got data suggested that HCV is implicated in the clonal selection of B-lymphocyte producing cryoprecipitable RF in type II MC[14]. **Manns** studied autoimmunity and hepatotropic viruses and stated that virus C clearly triggered an autoimmune reaction in genetically susceptible individuals [15].

Further studies are needed to correlate the decline in CD81 and CD5 B lymphocytes to the log decline in HCV RNA after INF therapy to help predict sustained viral response and to correlate the degree of their expansion to degree of liver fibrosis .

5. Conclusion:

We concluded that the overexpression of CD81 and the expansion of $CD5^+$ peripheral B-cells in HCV infected patients may possibly play a role in the development of HCV associated autoimmunity and that IFN- ribavirin therapy down regulates cell surface CD81.

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