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A newly discovered controversial role of the neutralizing HCV antibodies in the relapse of hepatitis C virus

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Abstract

Despite all intensive efforts and widespread use of direct-acting antivirals (DAA), and their high sustained virological responses (SVR) rate and the clearance of hepatitis C virus (HCV) RNA from serum, the mechanism of late relapse is still unclear. This study introduces a controversial role of neutralizing antibodies, and their influence in HCV protection and reactivation, by coating the virus's structural and nonstructural protein particles and protects it from CD8+ cytotoxic T cells and antiviral drugs. A new ELISA screening assay was designed to detect this Ag/ nAbs complex in serum samples. In a pilot study comprised of 76 subjects, 66 of them were diagnosed with chronic HCV genotype 4, patients were divided into 5 groups, group I patients were treated with interferon, group II patients received Sofo/dacla, group III patients did not receive any antiviral drugs, as for group IV patients were negative for HCV RNA, group V included sera samples collected from healthy individuals. Samples were collected after 3, 6, 12 months and the results we obtained concluded that there is a marked association between the increase levels of circulating immune complex C1, C2, C3 and the clearance of HCV RNA from blood, also we identified significant correlation between the relapse of HCV RNA after achieving SVR for a long time and the diminish in levels of these immune complex. These results have important implications for the development of real therapeutic and prophylactic vaccine and also raise a great possibility for developing a serological screening method for monitoring HCV treatment.

Keywords: hepatitis C virus; neutralizing antibody; antigen/antibody complex; HCV viral antigens; CD4 + T-cell; CD8 + T-cell

Introduction

Hepatitis C virus (HCV) is a major cause of liver damage and hepatocellular carcinoma worldwide [1]. HCV establishes a chronic infection in the majority of cases. It has been classified into seven major genotypes, which differ by 30% at the nucleotide level, and this positive sense, single-stranded RNA virus has a capacity for rapid evolution of variant viruses during persistent infection. HCV contains 9.6-kb RNA genome that is translated as a single polyprotein, then this single polyprotein is cleaved by viral and host proteases into structural proteins (Core, E1, and E2), p7, and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [2]. Viral attachment and entry are mediated by the envelope glycoproteins, E1 and E2 [3, 4]. The structural proteins of HCV include the capsid (core) protein and two envelope glycoproteins E1 and E2 [5, 6]. Around 80% of individuals infected with HCV develop chronic infections, more than 2% of the globe is chronically infected. Infection is the main etiological agent of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Among the mechanisms that the virus exerts to persist infection are: down regulating expression of its glycoproteins on cell surface [7-9], thus reducing the possibility of antibody recognition and destruction of infected cells.

Cellular immune responses, involving both CD8+ cytotoxic T lymphocytes (CTL) and CD4+ T-helper cells, play an essential role in the control of HCV infection, as they do in other persistent viral diseases. Whereas CTLs are traditionally thought to be the main effector cells that eliminate HCV infected cells [10], it is clear that HCV-

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specific CD4+ T cells also play a critical role. CD4+ T cells can potentially act in multiple ways and are central to the initiation and maintenance of adaptive immunity [11]. It plays a role in providing help for CD8+ T cells by cytokine production and also the activation of antigen-presenting cells, but there are multiple other mechanisms of action including direct antiviral effects, a role in B cell maturation, and regulatory functions [12].

The neutralizing antibodies play an essential role in controlling chronic viral infections. These cells mediate antibody dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) or antibody dependent phagocytosis [13, 14]. Our level of understanding on the role of the neutralizing antibodies activity in controlling HCV replication is limited. Most HCV seropositive patients have viremia, and there is no correlation between clinical outcome and antibody response to any specific HCV antigen. When the body's immune system raises antibodies against host or foreign substances immune complexes comprise these neutralizing antibody/ antigen complex forms. Normally, insoluble immune complexes that are formed are cleared by the phagocytic cells of the immune system, but when an excess of antigen-antibody complex are presented, the immune complexes are often deposited in tissues [15, 16], where they elicit complement activation, resulting in a localized inflammation and a variety of tissue lesions of autoimmune diseases, exacerbating disease pathology [17]. We postulated that during the HCV infection the broadly neutralizing antibody that are generated exist in two forms, a bound form where it effectively mask a proportion of corresponding HCV structural and a nonstructural proteins Abs that prevents its elimination, controlling its activities and a free form which is a non-functional non neutralizing abs.

In this study we investigate the formation of different types of circulating antigen/ antibody immune complex (C1, C2 and C3) in the serum samples of patients infected with HCV and its role in HCV RNA reactivation and relapse after it achieves SVR for long time, also we investigate the role of the free formed free nAbs for E1, E2 and core antigens in the persistence of the viral infection.

Materials & methods

Human sera

This study was carried out on a total of 76 patients, 66 of them are diagnosed with chronic HCV genotype 4, all patients wrote consent of acceptance to participate in this trial, patients were divided into 5 groups, group I included 22 patients treated before with interferon/ribavirin for 48 weeks and did not respond to the therapy, group II included 24 patients received (Sofosbuvir 400mg and Daclatasvir 60mg) daily for 24 weeks, group III included 14 patients none of them had been previously treated with antiviral drugs, group IV included 6 patients negative HCV RNA and detectable anti HCV antibody, group V included 10 sera samples that were collected from healthy individuals with no history of any liver complications, undetectable anti HCV antibodies and negative HCV RNA by RT-PCR in their sera were included as negative controls. Serum samples were collected from all groups 3 times after 3, 6 and 12 month intervals for quantitative measuring of HCV RNA, circulating free anti-E1, anti-E2, and anti-core antibodies, C1 (E1 antigen/ bounded anti E1) complex, C2 (E2 antigen/ bounded anti E2), and C3 (c antigen/ bounded anti core antibody) complex.

Immune complex preparation

HCV antigens (Core, E1 and E2) of different concentrations (0.05 ng/ml, 1 ng/ml, 1.5 ng/ml) and its antibodies (anti c antibody anti E1, and anti E2) of concentrations ranges from 0.05 to 5 ng/mL of SIGMA-ALDRICH, were obtained to make a vitro serial concentration of agglutinate immune complex C1, C2 and C3 (antigen/ antibody). An equal concentration of antigens and its specific antibodies were mixed to allow for agglutination and precipitation, to obtain three specific different complexes structure, C1 (E1 antigen/ anti E1), C2 (E2 Ag/anti E2) and C3 (c antigen/anti core antibody) with these serial concentrations; 0.05 ng/ml, 0.5 ng/ml, 5 ng/ml,10 ng/ml,15 ng/ml, 20 ng/ml. Every concentration of antigen is incubated to its similar concentration of Abs, a polyethylene glycol was added to maintain the activity of the structural protein, then the mix were centrifuged at 2500 g for 10 min, the supernatant are poured and the obtained sediments were added to 3 ml of Freund's complete adjuvant to immunize the rabbits groups with these immune complexes to induce the production of IgG-anti complex antibody.

Immunized rabbits with the prepared complex peptide (C1, C2 and C3)

A 30 male New Zealand albino rabbits weighing 8-10 lb were classified into three groups A, B and C, every group contains 10 rabbits and every group of rabbits injected with specific complex. Group A were injected with complex C1, group B injected with complex C2 and groups C with complex C3. All the rabbits in every group were injected with 0.20 ml of its specific complex intramuscular for 45 days, after six weeks blood samples were collected from the rabbits and 30 ml of immune serum (150 mg) was obtained from every rabbit to isolate the IgG anti-complex antibody, were all rabbits of group A has IgG anti C1, group B IgG anti C2 and the group C has IgG anti C3 antibodies. Ammonium sulfate 40% saturated solution was used to precipitate the antibody, we centrifuging the precipitate at 15,000 rev/min for 5 min in a Beckman 152 Microfuge, the supernatant was removed and the precipitate was washed twice with half-saturated $(NH_4)_2SO_4$, then we carefully remove and discard the supernatant, we resuspend the precipitate with PBS, another centrifugation was done to remove any remaining debris to obtain the anti-complex antibody from sera of all rabbit in each group, we determined the IgG concentration (ng/ml) by estimating the absorbance reading at 280 nm. ELISA assay was prepared to detect C1, C2 and C3 immune complex in serum samples of infected patients in all examined groups. 96-well plates coated with IgG anti (C1, C2, C3) antibody that obtained from the rabbits blood to capture the different complexes (C1, C2, C3) in serum samples of infected patients with HCV.

Description: The ELISA assay kit was designed to measure the quantities of C1, C2, and C3 in serum samples of the infected groups. We designed the supplied components in every kit as follow: (1) ELISA plates kit components for detection of C1, included a-component A coated plate

with 50 µl anti C1 and component B: complex C1 standard calibrators (0.05, 0.5, 5,10,15, 20 ng/ml). (2) ELISA plates kit components for detection of C2, included a-component A: coated plate with 50 µl anti C2 and component B: C2 standard calibrators (0.05, 0.5, 5, 10, 15, 20 ng/ml). (3) ELISA plates kit components for detection of C3, included a-component A coated plate with 50 µl anti C4 and component B: C3 standard calibrators (0.05, 0.5, 5, 10, 15, 20 ng/ml).

The other kits components included component C: HRP-conjugated anti-rabbit IgG 25µl, component D: TMB solution A (3, 3', 5, 5'- tetramethylbenzidine) 15 ml, component E: TMB solution B (H_2O_2) 15 ml, component F: TMB stop solution 30 ml.

To investigate the presence of different immune complex C1, C2 and C3 in serum samples of HCV infected patients

We run the three ELISA assay to detect each specific complex in serum sample for all patients in each group (I, II, III, IV, V). We diluted the complex C1, C2 and C3 standard (Component B) and every examined serum sample with PBS to ten concentrations, then 100µl of diluted standard, samples were added to the specific well (Component A) of the three designed kits. Incubated at room temperature for at least 1h, dilute 10 µl of anti rabbit IgG (Component C) with 10.5 ml of diluted solution then we added 100 µl of HRP-conjugate to each well. Aspirated and washed four times, and then incubated at room temperature for 30 min, 100 µl TMB peroxidase substrate solution (Component E) was added to the wells, and then it was incubated at room temperature for another one hour, then and each well was estimated at absorbance of 450nm using an ELISA reader (Bioteck, USA). The mean OD readings of complex C1, C2, and C3 were significantly higher than ($P < 0.05$) which is considered positive among all infected HCV patients.

Detection of free neutralizing antibody of HCV (E1, E2 and core antigens) in serum samples of the infected patients

A device comprising three plates connecting to each other was used, were every plate coated with 50 µg/ml of specific antigen (Core, E1 and E2) to capture its specific circulating antibodies in the HCV positive sera. To prepare the immune reactive plate, 50 µg/ml of each specific antigen was added to adsorb in to the micro plates for 48 hrs at 4°C. Wells were washed with PBST, a 100 µl of each tested serum samples of HCV was added for every triple wells for all groups (I, II, III, IV, V), we incubated the plates at 37°C for 1:30 h and then we washed them, after that we incubated them with 100 ml rabbit anti-human IgG conjugated (HRP) at 37°C for 30 min. The immune complexes were detected by adding 100 ml of TMB substrate solution, the wells were washed, and then the plates were measured by using an ELISA reader (Bioteck, USA) at 450 nm absorbance. To distinguish between positive and negative HCV sera the cutoff value was calculated as the mean value of the OD of HCV 150 negative samples plus 2 standard deviations (SD) equal to 0.05 ng/ml.

Determining the HCV RNA levels by bDNA

Blood samples for all tested groups were collected three times within 48 weeks for HCV- RNA were to evaluate the quantitation of the HCV RNA in serum samples of infected subjects by the by Amplicor HCV version 2.0 (sensitivity limit, 15 viral copies/mL).

Results

Exploring the changes in the level of HCV- RNA quantitation viral load

In group I, sixteen patients showed a high viral count (10×10^5 copies/ml), four patients showed moderate viral count (5×10^4 copies/ml), and two patients showed mild viral count (10×10^3 copies/ml), all patients in group III showed moderate to high viral count as showed in Table 1. In group II all patients showed undetectable viral count in first, second and third samples except four patients redeveloped HCV RNA in serum at the end of 12 months, respectively, after achieving SVR for six months, their viral load reached from (5×10^4 copies/ml) to (10×10^5 copies/ml) in most of them, while HCV RNA levels were below the detection limits in all patients of group IV and control group in all the three obtained sera.

Table 1 HCV RNA among the studied groups.

HCV RNA		*Group I*	*Group III*	*p*
Level Mean ± SD	Log RNA ($x10^3$/mL)	5.2 ± 0.8	5.0 ± 1.0	^0.411
Grade n, %	Mild	4 (16.0%)	5 (20.0%)	&0.849
	Moderate	6 (24.0%)	4 (16.0%)	
	High	15 (60.0%)	16 (64.0%)	

Note: ^Independent t-test. & Fisher's Exact test; No significant difference between the groups I and III regarding HCV RNA.

Identifying the levels of anti- HCV (Core, E1 and E2,) antigens in all groups

The outcomes OD readings were obtained from ELISA assays for the titer of the free nAbs of anti-core, E1 and E2 antigens in the sera of all examined patients during the three successive samples, showed that, in group I patients which had low viral count (10×10^3 copies/ml) the levels of their circulating free nAbs was (1.2 - 3.5 ng/ml) while their reference range was (0.02 to 0.05 ng/ml), patients which had a moderate viral load (10×10^4 copies/ml) the levels of their circulating free nAbs was (5.5 - 12.3 ng/ml) and patients which had high viral count (10×10^5 copies/ml) the levels of their circulating free nAbs was (12.2-20.5 ng/ml), in group II all three samples readings denoted to a decreasing in the levels of the free nAbs (1.1 - 3.0 ng/ml) except for the 4 patients whom relapsed after they achieved SVR for 6 months, their last sample showed an increase in the levels of free nAbs that reached up to (5.7 - 8.9 ng/ml), in group III were all of them had not been previously treated with antiviral drugs the levels of the free nAbs was (4.8 - 7.7 ng/ml), in 40% of them, and (7.0 - 11.9 ng/ml) in the remaining 60%, patients in group IV showed slightly positive for anti E2 and core antigens with a value ranged from (0.07-1.1 ng/ml), while in group V all the examined three samples showed a complete undetectable anti-E1, E2 and core antigens during the study course (Figure 1).

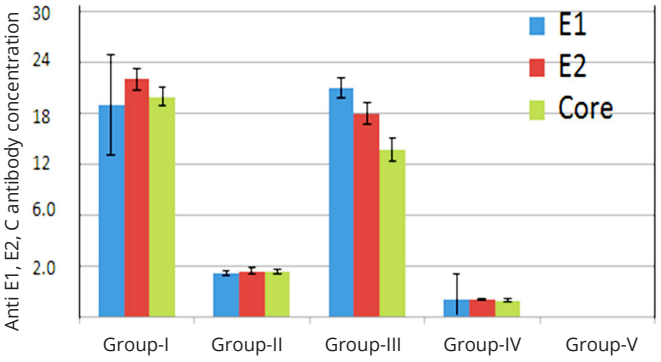

Figure 1 levels of anti- HCV (E1, E2, and core) among the studied groups.

Investigating the quantity of immune complex C1, C2 and C3 in the sera samples of all infected groups

The outcome measures revealed that patients in group I and III with high viral load of serum HCV RNA, the levels of C1 was 0.6-2.1 ng/ml, C2 was 0.7- 1.6 ng/ml and C3 was 1.1- 2.6 ng/ml, patients with moderate viral count their C1 was 2.2-5.1 ng/ml, C2 2.7- 4.6 ng/ml and C3 was 3.1- 7.0 ng/ml, while patients with mild viral load their C1 was 5.7-9.0 ng/m, C2 was 6.1-10.2 ng/ml and C3 was 4.8-9.5 ng/ml. In patients of group II the quantity measure of the immune complex of the three successive samples revealed that C1 was 7.6-11.6 ng/ml, C2 was 8.2- 13.0 ng/ml and C3 was 9.4 -14.8 ng/ml except the 4 patients which showed a relapse after 48 weeks the levels of their three complex showed severe decrease in the level of their complex, were C1 was 0.9-1.2 ng/ml, C2 was 1.0-1.8 ng/ml and C3 was 1.2-2.1 ng/ml (Figure 2), in group IV all the results showed undetectable absorbance readings for C1, C2 and C3 in comparing its results with the group V, the results that shows the levels of anti- E1, E2, c and the immune complex C1, C2, C3 regarding the levels of HCV copies (Tables 2 and 3) (Figures 3 and 4) (NB. The patients considered positive if their OD reading yielding absorbance over 0.05 ng/ml).

Table 2 Anti- HCV (E1, E2, and core) and complex C1, C2 and C3 in HCV RNA grades among the studied groups.

Group	*Anti- HCV antigens*			*Complex*		
	E1	*E2*	*Core*	*C1*	*C2*	*C3*
Group I						
Mild	5.4 ± 0.9a	6.8 ± 0.4a	7.4 ± 2.5a	7.1 ± 0.5a	6.3 ± 1.0a	6.6 ± 0.8a
Moderate	6.9 ± 0.5b	7.8 ± 0.5a	7.2 ± 0.2a	5.0 ± 0.7a	4.84 ± 0.8a	4.8 ± 0.6a
High	9.7 ± 2.4c	10.7 ± 2.7b	9.6 ± 2.3b	1.8 ± 0.9b	1.6 ± 0.9b	2.5 ± 0.7b
p	< 0.001*	< 0.001*	0.002*	0.001*	0.001*	0.002*
Group III						
Mild	3.4 ± 1.7a	3.7 ± 1.6a	3.3 ± 1.1a	6.7 ± 0.6a	6.6 ± 1.1a	6.9 ± 0.6a
Moderate	4.5 ± 1.8a	4.7 ± 0.7a	4.1 ± 0.5a	4.4 ± 0.2a	5.0 ± 0.7a	4.7 ± 0.8a
High	8.6 ± 2.0b	10.4 ± 2.6b	8.9 ± 2.2b	2.1 ± 0.8b	1.8 ± 0.7b	2.3 ± 0.7b
p	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*

Note: Data were presented as mean ± SD. All measured in OD, ANOVA test with pot hoc Tukey test-homogenous groups had the same symbol (a, b, c): E1, E2 and c were significantly highest in high viremia, followed by moderate, and least in low vireamia, C1, C2 and C3 were significantly highest in low viremia, followed by moderate and least in high viremia.

Table 3 Anti- HCV (E1, E2, and core) and complex C1, C2 and C3 among the studied groups.

Group	Anti- HCV antigens			Complex		
	E1	E2	Core	C1	C2	C3
Group I	8.3 ± 2.0a	9.4 ± 2.2a	8.7 ± 1.8a	4.0 ± 1.8c	5.5 ± 2.8c	5.2 ± 1.6c
Group II	1.7 ± 0.4b	1.8 ± 0.6b	1.8 ± 0.4b	8.8 ± 1.2b	9.9 ± 1.0b	10.6 ± 1.8b
Group III	7.2 ± 2.0c	8.2 ± 2.2c	7.9 ± 2.2c	4.3 ± 1.2c	5.0 ± 1.3c	5.6 ± 1.1c
Group IV	0.7 ± 0.3b	0.7 ± 0.2b	0.6 ± 0.2d	Undetected	Undetected	Undetected
Group V	Undetected	Undetected	Undetected	Undetected	Undetected	Undetected
p	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*

Note: Data were presented as mean ± SD. All measured in OD, ANOVA test with pot hoc Tukey test-homogenous groups had the same symbol (a, b, c, d): E1 and E2 was significantly highest in group I, followed by group III, then group II and least in group IV with no significant difference between groups II and IV. C1, C2 and C3 were significantly different among the studied groups; were the same in group I, III and highest in group II.

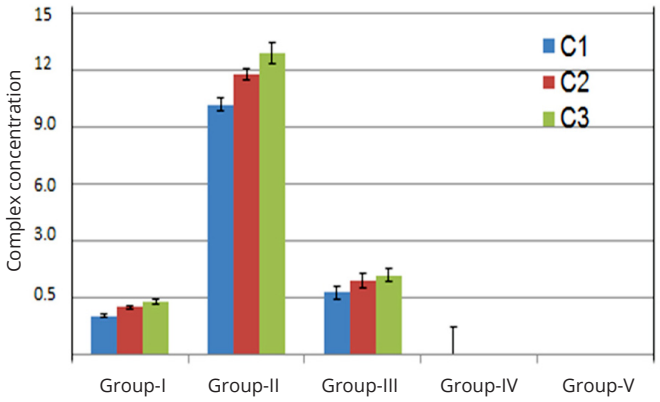

Figure 2 Levels of immune complex C1, C2 and C3 among the studied groups.

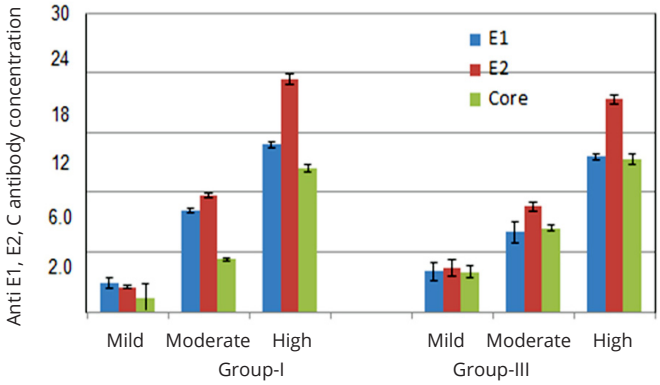

Figure 3 Levels of anti- HCV (E1, E2, and core) in HCV RNA grades among the studied groups.

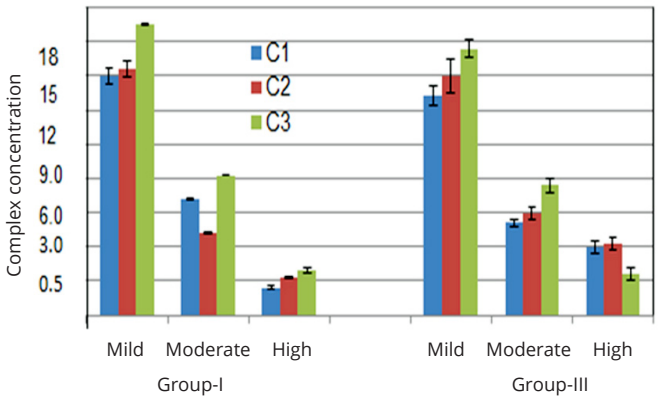

Figure 4 Levels of immune complex C1, C2 and C3 in HCV RNA grades among the studied groups.

Correlations between the quantity of free anti-E1, E2, core antibody and the complex C1, C2, C3

A strong positive correlation between increasing in quantity measure of circulating immune complex C1, C2, C3 and the decreasing in the level of free neutralizing anti- HCV (E1, E2 and core antigen) antibody, a decreasing in the level of these immune complex C1, C2, C3 showed in opposite a marked increase in levels of these free nAbs, this relation clearly explains the role of the neutralizing antibody which is generated by the B-cells during HCV infection, where the difference in their levels determines a state of a viral infection whither its acute or chronic, during the acute state the free broadly neutralizing antibodies are high, reflexing the inability of the viral antigens to attract the all produced antibodies, while in chronic state the viral antigens succeed in attracting most of the neutralizing antibodies causing a decrease in free Abs form and increase in bounded antibody form (C1, C2 and C3) (Figure 5).

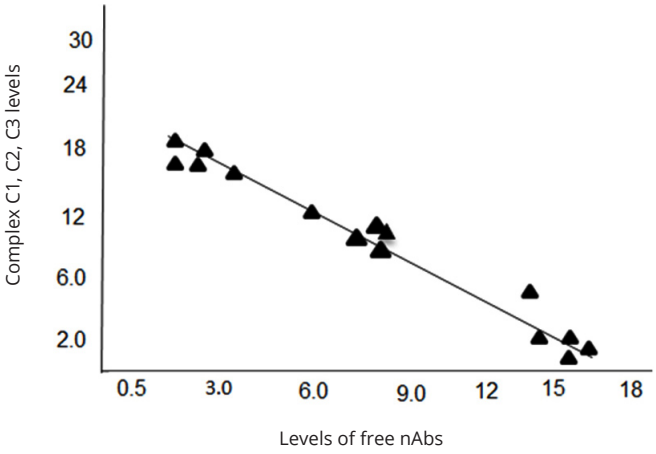

Figure 5 Correlation between levels of circulating immune complex C1, C2, C3 corresponding to the level of the free neutralizing anti- HCV (E1, E2 and core antigen) antibodies, the results reveals a strong positive correlations between levels of both parameters.

Correlations between anti-E1, E2, core antibody and the viral load

The obtained results for all examined sera of all infected HCV patients during the three successive samples revealed a positive correlation between increasing in viral load of serum HCV RNA and the increase in level of the neutralizing free anti- HCV (E1, E2 and core antigen) (Figure 6), also decreasing in the level of the viral count and marked decrease in levels of these free nAbs.

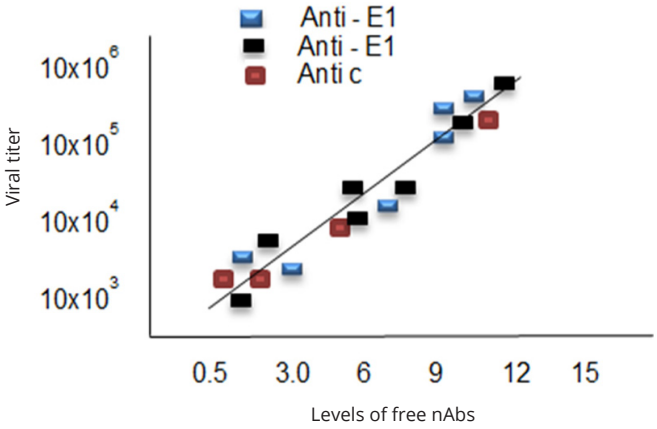

Figure 6 Shows the direct relation between increasing in viral copies in serum samples and the increase in level of the free nAbs (anti- E1, E2 and core antigen), respectively with decreasing in the level of the viral copies, a marked decrease in levels of free nAbs.

Correlations between the quantity of complex C1, C2, C3 and the viral count

Investigating the last correlation between the levels of the circulating immune complex C1, C2, C3 and the viral count a positive correlation between the decrease in quantitative measure of the immune complex and the increase in the titer of viral count, consequently a decrease in viral count followed by an increase in the levels of circulating immune complex (Figure 7).

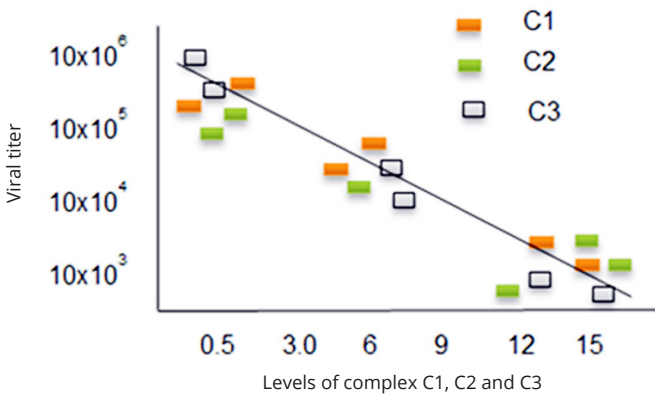

Figure 7 Shows the relation between the number of viral copies corresponding to the quantity of circulating immune complex C1, C2 and C3 in all examined infected patients, a strong correlation was detected between the decreasing in viral copies/ml (below detection limit or a mild viremia), and the increasing in concentration of circulating immune complex.

Discussion

HCV is a major national health problem in Egypt due to its high prevalence and associated morbidity and mortality. The Egyptian Ministry of Health is making a great effort to treat the HCV patients and is also conducting a medical survey to identify unknown infected patients. HCV consists of a lipid membrane envelope with embedded 2 glycoproteins; E1 and E2, serving in viral attachment and entry into the cell. The hypervariable region 1 (HVR1), found on the E2 glycoprotein shields the virus from the immune system and prevents CD81 from latching on to its respective receptor on the virus. In addition, E2 can shield E1 from the immune system. Within the envelope is an icosahedral core; core (C) protein, inside which the viral RNA is located. Treatment of HCV had evolved from interferon alpha (IFN-a), ribavirin with INF-a, to a newer era of DAA agents. However, despite successful treatment options for HCV which could achieve over 90% sustained virological response (SVR); undetectable HCV PCR six months after the end of therapy, a possible late relapse or reinfection still exist. While the current end point of therapy depends on undetectable HCV PCR, all patients remain positive for HCV broadly neutralizing antibody (nAb). Referring to HIV; another RNA virus, patients also remain positive for HIV Ab and deteriorate despite undetectable HIV RNA during antiretroviral therapy (ART). Thus elimination of viral RNA does not equal cure from it. Also, it is still unclear till now if the HCV nAb which is formed against the core and E1, E2 envelope glycoproteins, play a protective or a pathogenic role in HCV infection.

We assume that hepatocytes may be elected to originate this virus from itself and for itself and thus it learned how to protect it, so infected hepatocytes send an immunological signals for the B-cells to produce a broadly specific nAbs for every structural and nonstructural proteins parts of HCV, their nature may reflect the immunity of liver cells. This Abs can negatively inhibit or positively stimulate both of CD8+ CTL and CD4+ T-helper cells depending on the level of hepatocytes immunity. Immunocompromised hepatocytes activate the negative nAbs to attract the HCV antigenic structural and nonstructural proteins (E1, E2, and core) in a complex form protecting and controlling viral antigenicity for a long time. On the contrary, immunocompetent hepatocytes activate positive nAbs to stimulate both the CD8+ CTL and the CD4+ T-helper cells to clear the HCV RNA. This explains spontaneous viral clearance during acute infection in some individuals while others develop long-standing persistent infection. The results of this study support our hypothesis. Patients failed on IFN/ ribavirin and those with positive HCV Ab and positive HCV PCR, we found viral load to have a positive correlation with free nAbs and a negative correlation with immune complexes C1, C2, and C3. Patients with mild viremia, have a high level of complex and low level of free nAbs. While patients with high viremia, have low level of complex and high level of free nAbs. Patients treated with Sofo/ dacla completely prove our hypothesis as during the 24 weeks of treatment they had a consistently negative viral load with a markedly high level of immune complexes and a low level of nAbs. After 48 weeks post-treatment, four relapsers had increased viral load and free nAbs with decreased immune complexes than their previous readings and to make sure that the incidence was a relapse not a reinfection patients were asked if they were exposed to blood transfusion, blood dialysis or any medical or dental operations and they were clear. Patients with spontaneous viral clearance; negative viral load and positive HCV Ab, have a completely negative immune complex and a slightly positive free nAbs. So a positive immune response leads to complete clearance of the virus and its antigenic parts. This returned us to our opinion about the difference between the positive and the negative immune response. Current anti HCV medications simultaneously clear the viral RNA and stimulate the negative immune response to form an immune complexes with the viral structural and nonstructural proteins to protect and hide viral antigenicity for a long time. This mechanism introduce a mask results about the reality of the viral existence and clear answer about this huge positive results which reached to 90% with IFN-a and the newer direct-acting antiviral agents also it explain the reasons behind the incidence of hepatocellular carcinoma (HCC) and liver inflammation among patients with HCV-associated cirrhosis who received DAA agents [18, 19]. We are convinced that our results still needs sufficient evidence and we recommended extra studies to investigate the nature of these negative and positive antibodies.

Conclusion

These results carries an important implications for the development of real therapeutic interventions that depends on dissociating this immune complex to facilitate its eliminations and engulfing with our positive immune cells and also it raises the possibility of developing serological assays for monitoring HCV treatment.

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Authors' contributions

Doctor Sherif out most of the experiments and drafted the manuscript, Doctor Haider participated in the design and conceived of the study, Doctor Ehab acts for thoughtful

discussion and critical review of the manuscript and Doctor El-Hariri acts all the statistics and results revision.

Ethical approval

The animal study was performed in accordance with regulation and guidelines of the Institutional Animal Care and Use Committee of the Cairo University.

Competing interests

The authors declare no competing interests.

Abbreviations

E1: HCV envelope glycoproteins; E2: HCV envelope glycoproteins; c: HCV core antigen; nAbs: neutralizing antibody; Ag/Abs: antigen/antibody complex; IFN-a: type I interferon-a; Sofo/dacla: Sofosbuvir and Daclatasvir; DAA: direct-acting antivirals; C1: E1 antigen/ anti E1; C2: E2 Ag/ anti E2; C3: c antigen/anti core antibody; SVR: sustained virologic response.

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