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ASSOCIATION BETWEEN COMPLEMENT COMPLEX C5B-9 WITH RHEUMATOID ARTHRITIS IN AN IRAQI POPULATION

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ABSTRACT

Purpose: Investigating the level of concentration of complement complex C5b-9 in RA patients and controls serum. **Methods:** allow 2 ml blood samples to clot for 2 hours at room temperature or overnight at 4 C after that the samples were centrifuged for 15 min at 1000xg. Then, the the supernatant was collected and separated in plastic plain tube at deep freeze (-20 C˚) to be used for determine the concentration of complement complex C5b-9 in the serum of cases and controls. The complement complex C5b-9 concentration levels were investigated in 55 RA patients and in 32 healthy controls by using ELISA test. **Results:** The complement complex C5b-9 concentration levels were investigated in 55 RA patients and in 32 healthy controls. There is no significant difference when the mean of concentration level is compared in patients sera with the mean of concentration level in control sera. When the complement complex is used as a diagnostic test according to ROC curve, it appeared not beneficial for the diagnosis of RA when compared with Rheumatoid Factor (RF) and ACCPs (Anti Cyclic Citrullinated Peptide antibodies). **Conclusion**: there were many laboratory diagnostic tests to the diagnosis of RA like CRP, RF and ACCP but the ACCP antibodies test was more specific for predicting and/or diagnosing RA. and the Complement complex C5b-9 was not beneficial for prediction the diagnosis of RA. there is a significant difference when the mean of complement complex is compared according to positive and negative RF test among cases with RA.

INTRODUCTION

Rheumatoid arthritis: is a chronic systemic autoimmune disease which is typically present in adulthood, more common in women than men and affecting ∼1% of the adult population. in such disease many body joints are inflamed and a significant loss of function is caused by rheumatoid arthritis, roughly a quarter of patients cannot work due to the disability within 2 to 3 years of diagnosis. The design of joints affected is usually symmetrical, involving the hands and other joints. (Anderson *et al.*, 2012).

Rheumatoid arthritis is two to three times more common in women than in men, women with RA have an augmented risk for premature delivery. They are also more likely than healthy women to develop high blood pressure through the last trimester of pregnancy. For many women with RA, the disease goes into remission during pregnancy but the disease recurs and the symptoms can increase in severity after delivery (Firestein *et al*., 2012).

Rheumatoid arthritis is considered a common autoimmune disease. In these diseases, the body's immune system mistakenly attacks and destroys healthy joints, cells and tissues. The destruction mediated by immune cells of the joint architecture is associated with progressive disability, systemic complications, early death, and socioeconomic costs. (McInnes *et al*., 2011).

There are strong links between the pathogenesis of RA and the complement system (Okroj *et al*., 2007).

The complement system is a fundamental part of the innate immune defense, but it is also involved in the induction of the adaptive immune response and in the removal of waste, including immune complexes and dead cells. Also, it helps or complements the ability of antibodies and phagocytic cells to clear pathogens or antigens from an organism. (Janeway *et al*., 2001).

The complement system consists of a series of proteins more than 30 proteins found in the blood, generally synthesized by the liver, and normally circulating as inactive precursors (pro-proteins) (Abbas *et al.*, 2010). When stimulated by one of several triggers, proteases in the system cleave specific proteins and initiate an amplifying cascade of further cleavages. Finally, the end of this activation cascade is a massive amplification of the response and activation of the cell-killing membrane attack complex (MAC) that is (C5b-9) (Arumugam, 2004).

All three pathways of complement system merge at C3, which is then converted into C3a andC3b. The further formed C5 convertase from C3b cleaves C5 into C5a and C5b. C5b with C6, C7, C8, and C9 complex to form the membrane attack complex (MAC) C5b-9, which is inserted into the cell membrane, forms a hole in the membrane, and starts cells lysis (Basta, 2008).

The local complement production and activation may play an essential role in RA and specific modulation and inhibition of local complement production could be an attractive therapeutic target for RA. Rheumatoid arthritis is a severe long-lasting autoimmune disease characterized by inflammation of synovial tissue (ST) in joints, tendon sheaths, and bursae, which causes pain and dysfunction. The results to destruction of these structures and the treatment options are still limited to symptomatic and nonspecific immunosuppressive therapies. RA is often considered a predominantly T cell–related disorder (Miossec *et al.*, 1998), and as in other T cell– driven processes (e.g., experimental allergic encephalitis (Davoust *et al*., 1999)

There is accumulative evidence for an important role of components of the complement cascade in the pathophysiology of RA. Generally, the complement cascade is involved in the induction and progression of inflammation reactions and is a main defense system against various pathogenic agents, including bacteria, viruses, and other antigens (Speth *et al*., 1999).

The terminal complement complex (TCC), however, presented a design similar to that of C3. The significance of this noticeable differential distribution remains unclear, but it may reveal the level of tissue destruction and inflammation in a given patient sample, aside from other factors. However, production and activation of complement appears to be a particular feature in RA rather than OA, since none of the complement components examined showed intense expression in OA synovium. (Neumann *et al*., 2002).

The complement is present in synovial fluid, plasma and serum and is partly produced locally and partly originates from the circulation (Elena Neumann, et al ,2002). Complement levels in the synovial fluid of healthy person is higher than in patients with RA because of local consumption. So, the complement activation products such as sC5b–9, Bb, C3a, and C1 inhibitor–C1s and C1q–C4 complexes increased in synovial fluid of RA patients and complement depositions in synovial tissue from RA patients can be visualized by immunohistochemistry (Rincón *et al*., 2005).

The complement activation fragments are elevated in plasma and synovial fluid in patients (Morgan *et al*., 1988) and such complement activation products are found in joint tissues leading to the suggestion that joint damage is to some extent mediated by complement (Kemp *et al*., 1992).

Materials and methods: Human TCC C5b-9(Terminal Complement Complex C5b-9)

Table (1): components of complement kit.

Item	Specification	storage
Micro Elisa plate	8wells*12strips	4c
Reference standars	2 vials	4c
Reference standars and sample diluent	1 vial 20 ml	4c
biotinylated detection AB diluent	1 vial 10 ml	4c
Concentrated HRP conjugate	1 vial 120 µl	4c shading light
HRP conjugate diluent	1 vial 10 ml	4c
Concentrated wash buffer (25x)	1 vial 30 ml	4c
Substrate reagent	1 vial 10 ml	4c shading light
Stop solution	1 vial 10 ml	4c

This test is performed by using ELISA kit

A-Principle

This ELISA kit uses sandwich ELISA as the method. The micro ELISA plate provided in this kit has been pre-coated with antibody specific to TCC C5b-9. Specific standards or samples are added to the appropriate micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection of antibody was used specific for TCC C5b-9 Avidin –Horseadish peroxidase (HRP).

Conjugate is added to each micro plate well successively and is incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain TCC C5b-9, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme- substrate reaction is terminated by the addition of a sulphuric acid solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450nm ± 2nm. The OD value is proportional to the concentration of TCC C5b-9. We can calculate the concentration of TCC C5b-9 in the samples by comparing the OD of the samples to the standard curve.

B-Procedure

All reagents and samples were brought to room temperature before use. Centrifuge the sample again after thawing before the assay. All the reagents should be mixed thoroughly by gently swirling before pipetting.

Avoid foaming. It is recommended that all samples and standards be assayed in duplicate.

1- A 100 ml of standard, blank, or sample were added per well. The blank well was added with reference to the standard and sample diluent. Solutions were added to the bottom of micro ELISA plate well. Mix it gently. Cover the plate with sealer we provided. Incubate for 90 minutes at 37 c.
2- A 100 ml of biotinylated detection AB working solution was added to each well and cover the plate with sealer. Gently tap the plate to ensure through mixing. Incubated for 1 hour at 37c.
3- Each well was aspirate and washed , the process was repeated 3 times. Wash by filling each well with wash buffer (approximately 350 ml) (automated washer needed). Completing the removal of liquid at each step is essential. After the last wash, remove the remained wash buffer by aspirating or decanting. Invert the plate and put it against a thick clean absorbent paper.
4- A 100 ml of HRP conjugate working solution was added to each well. Covered with the plate sealer and incubated for 30 minutes at 37 c.
5- The wash process was repeated for 5 times as conducted in step 3.
6- A 90 ml of substrate solution was added to each well, covered with new plate sealer, incubate for about 15 minutes at 37c and protected the plate from light. The reaction time can be shortened or extended according to actual color change but not more than 30 minutes. When the apparent gradient appeared in standard wells the user should terminate the reaction.
7- A 50 ml of stop solution was added to each well. Then the color turned to yellow immediately. The order to add stop solution should be the same as the substrate solution.
8- The optical density (OD VALUE) was determined of each well at once, using a micro – plate reader set to 450 nm The users opened the micro – plate reader in advance, pre heated the instrument and set the testing parameters.
9- All the unused reagents back were putted after experiment into the refrigerator according to the specified storage temperature respectively until their expiry.

RESULTS

Detection of the difference in mean of serum C5b-9 concentration level in cases and control

In our study we investigate the serum concentration level of C5b-9 in 55 patients of RA and 32 control.

Table (2): Difference in mean serum complement complex C5b9 among cases and controls.

Serum complement complex C5b9	Healthy controls (n=32)	Cases with RA (n=55)	p	Difference in mean	Cohen's d
Range	(214.6 to 2784.9)	(47.2 to 2836.9)	0.24[NS]	-221	-0.26
Mean	1571.4	1350.4			
SD	853.9	829.7			
SE	151	111.9			
N	32	55			

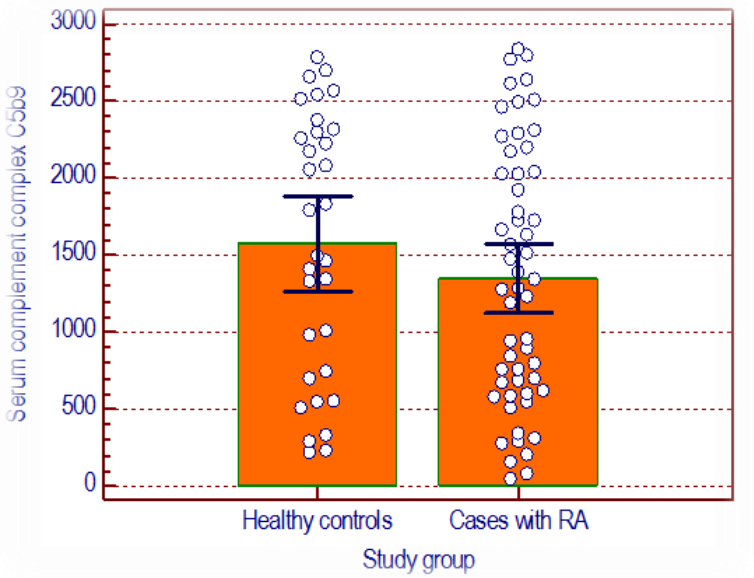

Figure (1): Dot diagram with error bars showing the case-control difference in mean (with its 95% confidence interval) serum complement complex C5b9.

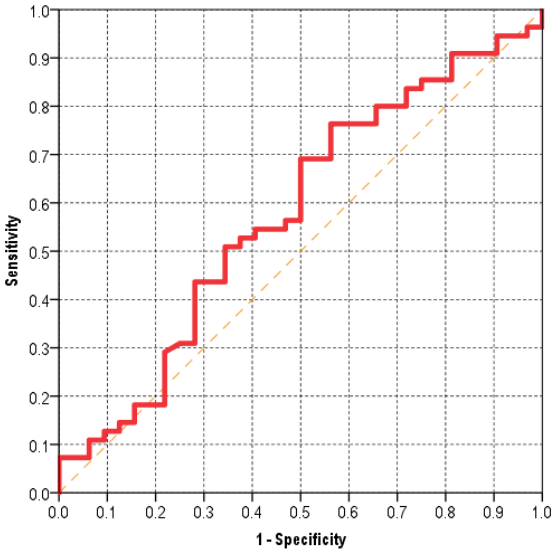

Figure (2): ROC curve showing the trade-off between sensitivity (rate of true positive test results) and 1-specificity (rate of false positive test results) for serum complement complex C5b9 when used as a test to predict a diagnosis of RA differentiating it from healthy controls.

This figure shows the complement C5b-9 is not benefitial as a test for predict the diagnosis of RA.

Determine the best tests according to ROC curve for predict the diagnosis RA:

Table (3): ROC curve for serum complement complex C5b-9 used to predict a diagnose of RA differentiating it from healthy controls.

	AUROC	P value
Serum complement complex C5b9	0.577	0.23[NS]

Table (4): ROC curve for selected tests used to predict a diagnose of RA differentiating it from healthy controls.

	AUROC	P value
CRP	0.653	0.013
RF	0.831	<0.001
ACCP	0.925	<0.001

***Area under ROC curve (AUROC)**

Sensitivity and specificity of values of complement complex in sera.

Table (5): Validity parameters for serum complement complex C5b-9 when used as a test to predict a diagnosis of RA differentiating it from healthy controls.

Positive if < cut-off value	Sensitivity	Specificity	Accuracy	PPV at pretest probability =		NPV at pretest probability =
				50%	90%	10%
207.8(Highest specificity)	7.3	100.0	41.4	100.0	100.0	90.7
1782.6(Optimum cut-off)	69.1	50.0	62.1	58.0	92.6	93.6
2777 (Highest sensitivity)	96.4	3.1	62.1	49.9	90.0	88.6

Association of complement complex C5b-9 with gender, CRP, RF and ACCP

This table shows that there is no significant difference when the mean of concentration of complement complex is compared in patients serum of male and female and also no significant difference according to the positive and negative tests of CRP and ACCP tests but there is a significant difference when the mean of complement complex is compared according to positive and negative RF test.

Table (6): Mean of serum complement complex C5b-9 by selected explanatory variables among cases with RA.

	Serum complement complex C5b9					
	RANGE	MEAN	SD	SE	N	P value
Gender						0.63[NS]
Female	(47.2 to 2836.9)	1326.3	838.7	123.7	46	
Male	(289 to 2495.5)	1473.4	818.1	272.7	9	
CRP						0.69[NS]
Negative	(158.4 to 2836.9)	1284.5	902.3	212.7	18	
Positive	(47.2 to 2794.3)	1382.4	803.1	132	37	
RF						0.028
Negative	(47.2 to 2836.9)	1866.6	868.3	274.6	10	
Positive	(77.2 to 2769.7)	1235.6	785.3	117.1	45	
ACCP						0.87[NS]
Negative	(47.2 to 2836.9)	1305.7	1141.7	403.6	8	
Positive	(77.2 to 2794.3)	1358	780.6	113.9	47	

This table shows that there is no significant difference when the mean of concentration of complement complex is compared in healthy control serum of male and female and also there is no significant difference according to positive and negative tests of CRP and RF tests.

Table (7): Mean of serum complement complex C5b-9 by selected explanatory variables among healthy controls.

Serum complement complex C5b9						
	RANGE	MEAN	SD	SE	N	P value
Gender						0.08[NS]
Female	(231.4 to 2784.9)	1443	795.3	156	26	
Male	(214.6 to 2695.9)	2127.6	949.2	387.5	6	
CRP						0.35[NS]
Negative	(214.6 to 2784.9)	1475.3	882.4	188.1	22	
Positive	(547.7 to 2657.2)	1782.7	789	249.5	10	
RF						0.5[NS]
Negative	(214.6 to 2784.9)	1532.1	874.2	165.2	28	
Positive	(1009.6to2657.2)	1846	735.9	368	4	
ACCP						***
Negative	(214.6to2784.9)	1571.4	853.9	151	32	
Positive	(*** to ***)	***	***	****	0	

DISSCUSION

Complement Complex C5b-9

In this study the concentration level of the complement complex (C5b-9) was investigated in serum of 55 patients with RA and 32 healthy controls.

The level of concentration is investigated by using ELISA kit and the results of this current study showed that the mean of patients concentration is (1350.4 pg/ml) Vs (1571.4 pg/ml) in control with no significant difference P (0.24).

The results of (Morgan *et al*., 1988) showed increased levels of C5b-9 in synovial fluid and plasma with rheumatoid arthritis when compared with controls also significantly elevated when compared with non-inflammatory or normal control plasma or synovial fluid.

Certain components of the complement are deposited in synovial tissues of RA patients. The levels of the complement in RA synovial fluid are often depressed, reflecting consumption, and the levels of cleavage fragments of complement components are commonly elevated. Therefore, the complement activation is essential in the pathogenesis of RA (James *et al*., 1991) and this explains the finding of the increased levels of complement cleavage fragments in the sera of patients with inflammatory Rheumatoid arthritis (Eleonora, 2011).

Also, TNFα seems to stimulate the complement system, as revealed by a reduction of complement cleavage products in the sera of patients treated with anti-TNF agents (Familian *et al*., 200). A mechanism that is proposed is that anti-TNFα decreases the plasma levels of CRP, which can activate the complement cascade through classical pathway. So, the reduction of the complement activation could contribute to the anti-inflammatory effects of anti-TNFα agents (Eleonora, 2011)

Association of Complement Complex with RF and CRP

Several factors can activate the Complement in RA, like rheumatoid factor, C-reactive protein which may be essential, since it is often increased in RA (Mallya *et al*., 1982) and can stimulate the classical pathway of complement Since different complement-activating

agents may be present simultaneously in vivo, and together may account for the total activation, it is not surprising that one of these factors (e.g., RF) may associate with activation in some patient subgroups while it does not in others (Mollnes *et al*., 1986).

In our research, we found a significant association when we compared between the mean of C5b-9 level concentration according to the presence and absence of RF in cases of RA, P value is (0.028). In control there is no significant difference. So in our study, the serum complement was significantly lowered in cases of active disease (RF positive).

The complement is traditionally considered to be mainly stimulated by bacteria or immune complexes. Rheumatoid arthritis patients have increased levels of circulating immune complexes; part of these complexes contains rheumatoid factors (RFs), which are autoantibodies against human IgG. RF-containing immune complexes that are able to stimulate complement via the classical pathway (Ballanti *et al*., 2011)

Association of C5b-9 with ACCP

In our research, there is no significant association when we compared the mean of C5b-9 concentration level according to the presence or absence of ACCP in cases of RA.

In the research of (Trouw *et al*., 2009), the results indicate that anti-CCP antibodies can stimulate the entire complement cascade, that C3a and C5a must be generated and show that anti-CCP can activate the human complement system via the classical and alternative pathways.

The research of (Clavel *et al*., 2008) demonstrated that, in vitro, anti-CCP antibodies interact with Fc receptors and stimulate immune cells. Taken together, these observations show that anti-CCP antibodies can trigger the 2 most prominent immune effector mechanisms used by antibodies.

And finally, the current research indicates a comparison between CRP, RF, ACCP and complement complex C5b-9 according to ROC curve which shows that the RF and ACCP are highly significant P <0.001 as the tests to predict a diagnosis of RA are differentiated from the healthy controls. The complement complex shows no significant difference as the tests to predict a diagnosis of RA.

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