Clinical Management of Patients with Systemic Lupus Erythematosus (SLE) with Different C1q-CIC and C3 Concentrations

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ORIGINAL PAPER

SUMMARY

Over the third of SLE (Systemic Lupus Erythematosus) patients have a high level auto-antibodies-antigen complex that contains some complement proteins, especially C1q as the trigger protein in the classical complement activation pathway. So, the SLE, as an autoimmune disease, is certainly related to disorders caused by activation of complement system, that finally leads to tissue damage. It may also be caused by hereditary deficiency (complement genes mutations). In such case, some components of the complement system might be inactivated. There are mutations that cause disorders in each of three complement system activation pathways (classical, alternative and lectin).The serum samples of SLE patients show the presence of specific autoantibodies for some complement components. Today, for clinical management of SLE patients, determination of level of C1q-CIC and C3 complement component in serum specimens have great diagnostic and therapeutic importance. During the year 2000, we analyzed a numerous serum samples from patients suspected to autoimmune diseases (SLE especially). The samples were collected from several clinics in the Clinical Center of University of Sarajevo, mostly from Clinic of Infectious Diseases, Pediatrics, Internal Medicine and Gastroenterohepatology Clinic. Primary samples went through screening for the presence of ANA using ANA-IFA method and further characterization of ANA positive samples was carried out using IFA-ANA titration, ELISA and nephelometry.

Keywords: SLE, C1q, CIC, C3, ANA-IFA, ELISA, nephelometry

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1. INTRODUCTION

Antinuclear antibodies or abbreviated ANA, are encoded by the corresponding genes located on HLA loci of a human chromosome 6, react with DNA, RNA, proteins and some ribo-nucleoprotein complexes in some pathologic situations. These nuclear antigens are also called auto-antigenes.

In the sera of patients suffering from systemic lupus erythematosus (SLE) first detected ANA were by IFA method. Limitation of this technique is ability to detect total ANA (tANA) but not specific characterization. Development of new methods with high level of sensitivity and specificity open the possibility to determine the different subgroups, or specific ANA (sANA) for individual nuclear autoantigenes.

Over the third of SLE (Systemic Lupus Erythematosus) patients have a high level auto-antibodies-antigen complex that contains some complement proteins, especially C1q as the trigger protein in the classical complement activation pathway. So SLE, as an autoimmune disease, is certainly related to the disorders of complement system activation, that leads to tissue damage due to accumulation of Circulating immune complexes (CIC). It may also be caused by hereditary deficiency of some complement components. The disease activity leads to specific complement auto-antibodies accumulation. Those are the reasons why, in clinical management of SLE, it is necessary to detect ANA and anti-ds-DNA as specific SLE risk factors as well as C1q-CIC and C3 quantitative determinations in serum specimens, regarding monitoring of SLE activity (1, 2).

Normal function of the complement system is to form specific attac protein complexes that damage the membrane of microorganisms. Even small damage caused by these agents is useful, since it can trigger metabolic changes in attacked cell and induce release of inflammatory agents. Other biological functions of complement are opsoniation and produce of different products, with hemolytic and anaphylactic properties (3).

Multimolecular antibodies-antigen complex or circulating immune complexes (CIC) could be find in healthy people as part of normal CIC clearing system. However, in some pathological conditions, like autoimmune diseases as Systemic lupus erythematosus (SLE), the concentration level of C1q-CIC increases in tissues and cause the activation of humoral immunity effector mechanisms, such as of classical complement pathway activation. The Accumulation of CIC in blood vessels or renal glomeruls can cause inflammatory processes and the induction of diseases such as thrombosis and glomerulonephritis (4, 5, 6).

The concentration level of C1q-CIC and C3 is important for therapy and monitoring disease activity in patients with SLE (7).

During 2009 and 2010 we have analyzed a total of 1320 serum specimens taken from patients suspected to SLE. First analyzes performed were screening using IFA-ANA and further semi-quantitative and quantitative analyses of 340 positive specimens (2+,3+,4+) by IFA-ANA titration and C1q-CIC-ELISA and C3 nephelometry as well. Other suspected patients with ANA positive results (border positive and 1+) had to be retested by screening IFA-ANA a month later.

2. MATERIAL AND METHODS

2.1. IFA-ANA

SPECIMENS STORAGE

Serum specimens must be stored at room temperature at least 8 hours before analysis. The specimens' storage could be for a period at 48 hours in refrigerator (+2 °C to +8 °C) and for a longer period at–20°C in freeze.

ASSAY PROTOCOL

All reagents must be kept at room temperature for half hour before use. 40X PBS concentrated buffer must be diluted in the ratio 1:40 with distilled water. In the procedure we used this PBS buffer for dilution of serum specimens.

A drop (20 -24 µl) of serum specimens, positive and negative control is added on slide wells with Hep-2 cells as substrate. After incubation for 30 minutes and washing with PBS buffer, a drop of fluorescing conjugate is added in each well. After 30 minutes of incubation, the slides are covered with mounting medium and observed by fluorescent microscope under 100X magnification objective and immersion oil. For correct results interpretation, the IFA-ANA positive and negative controls must be valid.

All clear positive serum specimens (++, +++, ++++) were analyzed by their titration to the end point dilution and by ELISA and nephelometry methods (8, 9, 10).

2.2. ELISA –TM-II-C1q-CIC (HYCOR)

For C1q-CIC concentration measurement in ANA positive serum specimens (++/ ++++) we used HYTEC automated ELISA system. HYTEC engine prepare all controls and specimen dilutions automatically. Before the procedure starts, it is important to wash all ELISA microplates (wells are coated with anti-C1q monoclonal antibodies) three times with diluted washing buffer (stable one week at 2-8 °C).

Dilution ratio between sample buffer and positive and negative controls must be 1:5 (50 µl sample buffer + 200µl controls).

Autostat™ II kits contain conjugate ready for use, substrate and stop solution. Diluted serum specimens that contain C1q-CIC, bind to antibodies on the ELISA microplate wells. After incubating at room temperature and washing out the unbound material, it has been added horseradish peroxidase conjugated anti-IgG monoclonal antibody that binds to the immobilized complexes. Further incubation and washing steps are followed by addition of the substrate (tetra-methyl benzidine substrate-TMB) to each well. The stop solution turns the color in ELISA microplate wells, from dark blue to yellow (11).

The color intensity is proportional to the amount of immune complexes present in the original serum sample.

Interpretation of results:

µg/ml	C1q CIC
Negative	<40
Equivocal	40 – 50
Positive	>50

2.3. NEPHELOMETRY

Total C3 serum concentrations have been measured using nephelometry method (Dade Behring Nephelometry type II-Siemens II; Dade Behring, Marburg, Germany). ANA positive serum specimens for analysis were either stored in refrigerator (+2°C to +8 °C) or for longer period in freezer on -20 °C. During nephelometric measurements, the intensity of scattered light is proportional to the concentration of relevant proteins in the serum sample. The reference interval values applied for serum and plasma specimens for C3 in adults are from 0,9 to 1,8 g/L. Complete procedure and result calculations ware automated (12, 13).

3. RESULTS

Screening of 1320 serum specimens for the presence of ANA is performed by ANA-IFA method and characterization of ANA positive samples (2+, 3+, and 4+) was carried out using IFA-ANA titration, ELISA and nephelometry.

ANA-IFA screening test had total incidence positivity of 63%. Among these ANA positive specimens, majority of them (65 %) were borderline and 1+ positive.

All borderline positive ANA and ANA 1+ positive samples were sent for retesting a month later.

A total of 462 serum samples, ANA 2+, ANA 3+ and ANA 4+, were titrated to their dilution end points in range of !:80 to 1:5120.

The majority of specimens had relatively high ANA titer, from 1:160 – 1:320 (351 or 76 %).

The high ANA titer from 1:640 to 1:2560 showed 51 specimens or 11,62%.

Only 6 specimens or 1,3% had extremely high ANA titer of 1:5120 and 11,08% or 54 specimens had low ANA titer (1:80).

The most samples showed homogeneous fluorescence type and only a few spickled types. IFA-ANA is still an indispensable method of screening and ANA results must always be taken into consideration with other relevant findings in SLE clinical management.

All IFA-ANA specimens, with 2+, 3+ and 4+ positivity (ANA titer ≥ 1:80), were also analysed using ELISA method and nephelometry for level determination for C1q–CIC and C3 The aim of the study was to determain correlation of immunological and inflammatory parameters between concentrations of circulating immune complexes (C1q-CIC) and C3 complement components and disease activity at SLE patients.

However we analyzed a total of 462 ANA positive serum samples (76, 4% female and 23,6% male) with quantitative ELISA C1q-CIC and C3 nephelometry methods.

The referral or normal values of C3 component using nephelometric measurements were between 0,9-1,8 g / l. All values greater than 1.8 ment positivity.

Referral or normal values for C1q-CIC, that were determined using ELISA method, were as follows:

- Negative findings < 40 µg / m
- Borderline positive = 40-50 µg /ml
- Positive > 50 µg /ml

Obtained results were:

- C3 positivity -5 samples (1, 08 %)
- C3-border positivity–7 samples (1, 58 %) (values greater than 1,6)
- C1q-CIC positivity–22 samples (4, 76 %)
- Borderline C1q-CIC positive–18 (3, 89 %)

Individual parameter values are represented in table 1.

parameters	woman	man
C3-min	0,050-0,367	0,536-0,543
C3-avr	1,110-1,520	1,110-1,510
C3-max	1,530-2,020	1,530-1,940
C1q-CIC-min	5,08-9,37	4,72-10,04
C1q-CIC-avr	10,01-49,90	10,04-55,05
C1q-CIC-max	50,80-131,6	55,30-145,0

TABLE 1. Obtained level values for C3 and C1q-CIC in serum specimens According to specific parameters (min-minimum; avr-average; max-maximum)

As shown in table 2. (special cases), only three serum specimens (1, 2 and 3) with extreme positive C3 and C1q-CIC values, were also positive after anti-ds-DNA and anti-Sm testing. Some other specimens with lower these values were negative to anti-ds-DNA or anti-Sm autoantibodies. Full evaluation of obtained results was performed in collaboration with clinicians according to SLE classification official criteria. These first three specimens satisfied four and more of official SLE criteria, while others did not.

4. DISCUSSION

Complement proteins (more than 25) makes app. 10% of serum globulin fraction. They are marked with C letter and the number represents their determination order. There are 7 serum, 5 membrane regulator proteins and 8 receptor proteins on the surfaces of appropriate cells. Most C proteins are synthesized in the liver, C1, D-factor and probably properdin in macrophages and T lymphocytes.

Complement system works as an interactive complex, which means that the initial activation reaction has the function of "domino effect", runs down the complement activation of protein components, to the final stage, when on the microbial pathogens surface or infected cells, by forming tubular channels composed of C9 molecules, causes destruction or lyzis of cells or pathogens. After initiation, the series of specific reactions lead to the synthesis of C3 convertase enzyme, that function as a complement protease. There are, well known, three pathways of complement system activation: classical, alternative and lectin. All of them imply inducing the synthesis of C3 convertase and the final product is a specific membrane-attack complex, causing destruction of infected cells or pathogens (14,15).

As part of the immune system, complement protein complex plays an important role in the following processes:

- Defense against microorganisms
- Organism clearing from immune complexes
- Transport of immune complexes to the liver for final degradation
- Stimulation of phagocytosis
- Opsonisation and production of different molecules with chemophylactic and anaphylactic properties

Sometimes, due to specific disorders, this complement system can be more or less dysfunctional. It can be the cause of serious diseases such as systemic lupus erythematosus (SLE), ovarian cancer and so one. It means that this complement pathological stage may cause serious disorder of circulating complexes clearing process and in the same time, their accumulation in tissues and induction of corresponding inflammatory processes.

In the SLE acute stage, the complement proteins, as well as acute reactants, are produced in increased concentrations and hence, the monitoring of their levels in serum of SLE patients, has diagnostic and therapeutic importance in clinical management of this disease (16,17).

Since the SLE frequency is ten times higher in women than in men, during the research it was monitored the level of C1q-CIC and C3 , by nephelometry and ELISA method, according to sex of patients.

Several serum specimens with extremely high levels of C1q-CIC and C3, had previously high levels of ANA, that indicated the active phase of the disease, so that IFA-ANA might be used as a good screening method in diagnostic process of suspect SLE patients. Very high levels of all three parameters (ANA, CIC-C1q and C3) have had some rare SLE cases indicating the disease activity.

specimen	C3-value	C1q-CIC value	Anti-ds-DNA	Anti-Sm
1	1,870	131,6	+	+
2	1,880	58,7	+	+
3	1,840	77,1	+	+
4	1,510	55,3	+	+
5	0,519	132,7	-	+
6	2,020	18,6	+	+
7	1,820	21,3	+	+
8	1,890	4,7	+	-
9	1,030	82,3	+	+
10	1,060	60,9	+	+
11	1,100	50,8	+	+
12	0,913	145,0	+	+
13	1,130	104,0	+	+
14	0,788	65,3	-	+

TABLE 2. Comparison of different of C3 and C1q-CIC values and presence of anti-ds-DNA and anti-Sm autoantibodies

However, SLE is an autoimmune disease that is accompanied by the presence of ANA and high levels of major complement components such as C1q-CIC and C3 when it comes to active phases of the disease as shown in our findings as well. Unclear aspect of SLE is molecular aspects of its pathoetiology although it is well known that disorder of complement system might be the important cause of disease appearance (18,19).

All this arguments suggests that C1q is a vital component of complement in the normal processing and clearing of immune complexes from the blood, certainly, in cooperation with the C3 component. It would be useful to supplement this research with the CH50 levels determination and molecular biological analysis of specific genes to establish whether our SLE patients have congenital disorders of C1q or C3 components or not. Despite, the results obtained with regard of monitoring the levels of C1q-CIC and C3 at SEL patients, represent an original contribution to knowledge of this issue, there is no designed method by which it could be possible to monitor the dynamics of all quantitative changes of complement components at the same time. Equally, as important of C1q component for their binding to CIC it is important C3 component for the transport of immune complexes to the place of their degradation, and any study of these two components, in scientific investigations, is valuable and useful (20,21).

C3 complement component is part of the complement system which contains at least 15 proteins that partic-

ipate in the entire series of reactions creating antigen-antibody complex. It is presents in many tissues: liver, macrophages, fibroblasts, lymph cells, skin. C3 complement component by the action of C3 convertase splits into C3a and C3b subunit. In some repeated infections, the C3 complement values are reduced. It was determined the correlation of SLE predictors, such as anti-ds-DNA with appropriate clinical picture of SLE patients.C3 component is the most important central molecule of complement system, because the both, classical and alternative complement activation pathway, means activation of C3 and that provides a processes of opsonisation and anaphylactic activation. C3 deficiency is transmitted as a recessive autosomal feature.

Patients with this disorder develop strong recurrent episodes of pneumonia, meningitis, and sepsis peritoneosa and glomerulonephritis in 15-20% cases. Monitoring of immature CD4+ T lymphocyte, as responsible factor for SLE development is important in clinical treatment of this disease (22, 23).

It is therefore very important, in SLE clinical management, to pay attention for specific symptoms of patients in which is measured elavated level of C1q-CIC and C3, due it can show much more about the pathological basis of disease. Secondary disorders of the complement system, that are not inherited, are caused by immune complexes and complement proteins involved in the process of their transport and decomposition. Disturbance of the complement system can be caused by nutritional factor (nutritional deficiency)(24).

As a complement implication is evident in the SLE pathogenesis, this problem is investigated by molecular biological methods as the expression of the specific complement genes level, that encode some complement components. Regarding to SLE ethiopathology today is applied PCR-RFLP method to perform specific mutations determination in the C1q gene at SLE patients. Up to date, there are five very well known C1q gene mutations:

- C → T–186 (Gln)–C1qA gene
- G → A–15 (Gly)–C1qB gene
- C → T–150 (Arg)–C1qB gene
- G → A–6 (Gly)–C1qC gene
- C → T–41 (Arg)–C1qC gene

Molecular basis of their association with SLE is still in the testing phase. For now, is still actual the multifactorial base of SLE pathoetiology, in which the main roles play environmental, genetic and hormonal factors. It should, also, be noted, that the homozygote nature of complement deficiency inheritance is closely associated with the possibility of the SLE appearance (25,26).

Anti-C1 test as SLE predictive factor is necessary to introduce in our diagnostic program. Our SLE patients had showed the presence of anti-Sm and anti-Sm/RNP that are, also, diagnostic criteria of great importance.

Due to evident implication of complement system in SLE, the pathoetiology of this disease is object of extensive investigation on the level of macromolecules information (RNA or DNA). By molecular-biological method such as PCR, RT-PCR, PCR-RFLP, DNA sequencing it is possible to determine, for a short time, with highest level of sensitivity and specificity, the specific complement gene mutations at SLE patients.

It is clear, that these methods must be included, as soon as possible, in SLE clinical management in B&H, together with PCR analysis of HLA-I, HLA-II, RF-5, Bank-1, MBL2 and MASP genes. Specific polymorphism of intron 4 incharge of programmed cell death (PDCD1) gene is strongly associated with increased lupus erythematosus appearance risk in familial and sporadic disease cases. As well, a low level of mannan-binding lectin (MBL) plasma protein is predicting SLE development and represent factor for respiratory tract infections and arterial thrombosis. Today, use of PCR and multiplex PCR methods made possible detection of MBL-2 gene specific pathological mutations (27,28,29,30,31).

Including this molecular approach will provide illumination of the important molecular etiopathological aspects of Systemic lupus erythematodes in B&H.

5. CONCLUSION

The ratio between male and female patients was 1:3,16. This fact confirms that the SLE is much more common in women due to their specific hormonal status. There are no significant differences regarding established C1q-CIC and C3 values between men in women. It was determined the SLE correlation predictors such as anti-ds-DNA with appropriate clinical picture and diagnosis of SLE patients. Anti-C1 test SLE as predictive has not been made, and it is necessary to be introduced in our diagnostic program. Anti-Sm and anti-Sm/RNP are important diagnostic criteria and were helpful in final clinical SLE management during our investigations. Extremely high ELISA- C1q-CIC (131, 6–145) and C3 values (1,940–2,020) determand by nephelometry were detected at SLE patients with active episode of this disease. Others patients had not SLE or probably incomplete SLE, but not in the active phase. As SLE specific risk factors, in further investigations must be included some aspects of SLE molecular biological, such as PCR analysis of HLA-I and II gene or RF-5 gene. The nature of complement system disorders can be investigated by PCR characterization of mbl-2 gene and masp-gene. This way it will be possible to determine whether disorder of complement system is in the activation of classical, alternative or lectin pathway.

List of Abbreviations

- IFA – indirect immunofluorescent assay
- ANA – antinuclear antibodies
- tANA – total ANA
- sANA – specific ANA
- C1q – complement component
- C3 – complement component
- CIC – circulating immune complexes
- ELISA – enzyme linked immunosorbent assay
- SLE – systemic Lupus erythematodes
- HLA – human leukocyte antigens
- TMB – tetra methyl benzidine substrate
- CH50 – Total Hemolytic Complement
- Anti-Sm – antibodies to Smith antigen
- Anti-Sm/RNP – antibodies to Smith antigen/ribonucleoproteins complex
- Anti ds-DNA – antibodies to double stranded DNA molecules
- PCR – polymerase chain reaction
- RT-PCR – reverse transcription PCR
- RFLP- restriction fragmenth length polymorphism

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