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CHEMILUMINESCENT TECHNOLOGY IN A LIVE IMAGE FOR DETECTING THE BLOOD BIOMARKERS OF AUTOIMMUNE DISEASES: BIOMARKER OF LESION DEPTH

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ABSTRACT

An analysis was carried out and a technology was proposed in connection with the use of complement components to identify biomarkers of autoimmune diseases. The technology of detection of a biomarker of systemic lupus erythematosus (SLE) - subisotype C4A5-pI-3,8-BLOT-pH7 of the complement system component C4 in the serum of patients is evaluated. The uniqueness of the identified subisotype lies in the indication of the depth of the patient's lesion with SLE against the background of antiphospholipid syndrome (APS). The prospects of biomarker subisotypes involving the complement system are discussed.

KEYWORDS: Real-time chemiluminescence, isoelectrofocusing in polyacrylamide gel, electric immunoblotting, immune enzyme analysis (IEA), components of C4A and C4B, biomarkers, systemic lupus erythematosus (SLE), antiphospholipid syndrome (APS).

ABBREVIATIONS

APS	Antiphospholipid syndrome
CIC	Circulating immune complexes
EPO	Erythropoietin(s)
FIEA	Functional IEA
IEA	Immune enzyme analysis
IEF	Isoelectric focusing

PAG Polyacrylamide gel
pH The value of the acidity of the medium
pI Isoelectric point
SLE Systemic lupus erythematosus

1. INTRODUCTION

Among hereditary and acquired diseases, a group of systemic rheumatic autoimmune pathologies is distinguished as multifactorial, proceeding through the appearance of chronic forms that are difficult to diagnose. The search for phenotypic biomarkers of rheumatic diseases such as systemic lupus erythematosus (SLE) by serum analysis, as in the case of other systemic rheumatic autoimmune diseases, is relevant.

The most complexly organized and effective protection of the first level of the body is the metabolic-cellular complement system of innate immunity. At the same time, a special role in early warning and complement action is played by the C4+C3 component system, activated fragments (C4b, C3b) of which covalently bind/cross-link carbohydrate and protein targets on the cell surface or in solution, exhibiting properties against pathogens and human altered/ pathological structures. Congenital and acquired deficiencies of the C4B and C4A isotypes of the C4 component of the complement system affect the functioning of all three complement pathways (lectin, classical and alternative) and allow us to assess not only the risk of rheumatic disease, but also its current status by the presence of circulating immune complexes (CIC) and protein aggregates associated with C4b in the blood.[1]

At the same time, the disease status may progress towards increasing the depth of damage to the body by emerging concomitant diseases. Thus, there is a task of searching for biomarkers not only as indicators of the presence of a particular disease, but also as indicators of the depth of the developing pathological process due to the contribution of additional types of pathologies.

1.1. Evaluation of approaches in the study of SLE biomarkers

*The use of combinations of isoelectrofocusing (IEF), polyacrylamide gel (PAG) and immunoblotting[2-4] does not provide unambiguous identification of biomarkers of rheumatic and neurodegenerative diseases, although IEF types of disease patterns are detected, including using affinity blotting for viral antigens and chemiluminescence analysis.

*Blood protein aggregates formed and distributed in PAG at strongly acidic pH[2,5], in cases of SLE, are characterized by an increased content of autoreactive CIC of the predominant IgM and/or IgG type, which also include C4/C3 complement components.[5-7] Such CIC can be considered as variants of SLE biomarkers, which are also defined with micropanel immune enzyme analysis (IEA).[8]

*Changes in the complement system are recorded as biomarker characterization processes of SLE[5,9,10,11], allowing to systematically describe types of pathologies, including SLE with antiphospholipid syndrome (APS) or concomitant other types of pathologies (hypertension and cisstatin therapy.[5,10,12] In cases of SLE+APS, such complement components as factor-H (Sia-binding lectin), mannan-binding lectins, as well as ACLA-IgM decrease in the blood, against the background of an increase in C4A, C4B and C3.[10] In rheumatic diseases, the levels of complement activation products with the participation of reactive C4b and C3b fragments (form covalent complexes and aggregates) increase, which correlate better with the disease.[11]

*Hereditary and acquired deficits of C4A or C4B (a geographical dependence of the deficiency was noted) in the assessment of rheumatic diseases, including SLE, are widely considered in the literature.[5,12-14] The deficits of the C4 component isotypes C4A and C4B were found to correspond to the risks of systemic diseases in patients.

*The potential of CIC biological activities in cases of SLE[15] includes, at least, the determination of the cytolytic activity of CIC against complement-labeled cells of foreign or human abnormal cells, as well as stimulation of cytokine production.

*In connection with vaccines[16], research on ways to reduce the risk of viral and bacterial infections in patients with SLE is of interest.

*Previously, we developed variants of the functional IEA (FIEA) of isotypes C4A and C4B (micropanel and blot – independent methods in detection that complement each other informationally). Blotting technology, as more sensitive and more informative in comparison with micropanel analysis (according to our data), is original (priority) and multi-stage (see below).

The technology of highly effective image analysis of protein/glycoprotein subisotypic pathological aggregates associated with antigens of molecules and fragments of subisotypes of C4B and C4A isotypes includes:

a) desialylation of patient sera by the original procedure (desialylated sample preparation);
b) IEF of serums in the PAG plate (IEF-PAG) in the pH gradient ranges 2-8;
c) semi-dry electrobloting on a sandwich membrane (obtaining BLOT-pH7 and BLOT-pH4),
d) treatment of blot with human C4 antibodies conjugated with peroxidase;
e) monitoring of the kinetics of blot chemiluminescence in the presence of a chemiluminescent peroxidase substrate.

The aim is to describe the proposed technology, including using the example of a biomarker we have established (isotype C4A included biomarker subisotype-5 as maximally acidic form pI 3.8: C4A5–pI-3.8-BLOT-pH7 [blot included final treatments at neutral pH]) in the blood serum of patients in cases of SLE+APS.

2. RESULTS AND DISCUSSION

A brief description of the generalized technology includes the choice of object and factors, selection of conditions, features, detail, priority, prospects in the SLE study, prospects for further development of procedures and the proposed technology itself is represented below.

2.1. The main constituents and features of the proposed technology

1) *Desialylation of patient sera*

The serums of patients who were examined at the Clinical Diagnostic Center at the *G.N. Gabrichevsky Institute* were studied. Desialylation was performed using our original method.[17] Desialylation eliminates the excessive negative charge of proteins that prevents the separation of IEF-PAG. Selection of optimized desialylation conditions (source of sialidase, time of contact with serum, temperature of reaction) sera of patients were carried out as a result of controlled images of the distribution of C4A and C4B isotypes in cases of model sera with a known deficiency of one of the isotypes (C4A or C4B, C4A>>C4B, C4B>>C4A) of the C4 component (according to the data of the micropanel FIEA variants for C4A and C4B early developed by us.[1]). The advantages are the possibility of using partially purified (crude) sialidase (Enzyme Classification 3.2.1.17) preparations, increasing the reproducibility of the results. The technology of FIEA–controlled desialylation developed by us has been used by us in the study of blood glycoproteins.

2) *IEF-PAG*

Horizontal semi-preparative IEF-PAG (glass plates 25x25 cm, gasket thickness more than 1 mm) was used instead of IEF in the agarose layer in the study of (sub) isotypes C4A and C4B.[17] The procedure was initially worked out using examples of erythropoietin (EPO) separation. It allows to use all the advantages of PAG instead of agarose (identify biomarkers in the gel thickness, conduct electroblotting). The procedure was successfully applied to the sera of patients with hereditary C4A or C4B deficits (a new pI region was found for registration of protein aggregates with C4 antigens, a more acidic region compared to the original C4A and C4B blood). Sera were separated by IEF-PAG in variants of the general pH gradient 2-8 [a mixture of pH gradients 2-4 and pH 4-8] ("kit 2.5-6.5", Amersham Pharmacia Biotech, Sweden; protein staining with fluorescent dye *SYPRO Protein Blot Stain*, Bio-Rad; *Methyl Red* (Marker Dye) pI 3.8, Sigma, USA; additional markers included a *set of forms of recombinant human EPO* separated by us with pI in the range 3-5). The pH gradient in the gel was controlled by testing extracts from a sequential set of gel samples along control tracks without or containing pI markers, as well as in tracks of sera samples between electrodes.

Electrophoresis was also performed in a vertical PAG plate (Bio-Rad Lab., USA) with sodium dodecyl sulfate in the Lemmli system, followed by electroblotting on a membrane sandwich. This makes it possible to estimate the molecular weights of protein aggregates due to the interaction of protein with detergent.

3) *Obtaining pH7 and pH4 blots*

Getting a pH7 blot

A standard procedure for semi-dry electrical blotting, tested in the case of EPO transfer from a gel. Nova prefix is used for a device (Pharmacia, Sweden) designed for horizontal electrophoresis in a PAG plate. For the first time, it is proposed to obtain and use photographs of a semi-dry (drying within few minutes) Durapore hydrophilic membrane (Durapore, Millipore) on both sides as tracing paper of tracks on the blot membrane, which allows increasing the number of tracks created in the gel plate and improving the visually controlled comparison of neighboring tracks, including control and experimental ones.

Obtaining a blot-pH4 (inter-blot semi-dry electrical transfer)

Electric blotting in an acetate buffer pH 4.5 was perfomed to improve the separation of subisotypes in the C4A and/or C4B region with increased hydrophobicity on the blot. The

perspective is a further assessment of the contribution of each subisotype (from a set of visible images of subisotypes) to the diagnosis of a multibiomarker disease.

4) *Treatment of the blot with human C4 antibodies labeled with peroxidase*

On the pH7 and pH4 blots, the C4A and C4B subisotypes were manifested by polyclonal antibodies to human C4 conjugated with peroxidase in working dilutions 2000-2500 times. Additional preparation and analysis of the blot was carried out at pH2.9 (inter-blot semi-dry transfer at pH 2.9). At the same time, further improvement of the background on the blot was achieved (reduction of the nonspecific glow of proteins, elimination of the inter-electrode gradient of the background). Electrical blotting was performed in 0.7% glacial acetic acid.

The original procedure proposed by us for transferring the resulting chemiluminescence pattern from a blot after IEF-PAG (blot-pH7/pH4) to a new (pure) blot. The substrate products of the reaction stabilize the peroxidase.

The prospect is to enhance the discreteness of protein images, including in cases of chemiluminescent "staining"/ manifestation of separated proteins with a labeled immune sandwich.

5) *Registration of the kinetics of the glow of blots in the presence of a chemiluminescent peroxidase substrate*

Peroxidase was manifested by *BioWest* chemiluminescent substrate (Pierce, USA), which differs from ECL, ECL+ with increased sensitivity and high stability (active 24 h at room temperature). It is possible to reduce the amount of substrate consumption by an order of magnitude.

Luminescence was recorded in the *BioChemi System* (UVP, USA) in an optimized live image (real time) mode. Chemiluminescence was registered in a variant of stepwise kinetics in a series of nonlinear exposures of the seconds-minutes scale (each exposure as a step). For the first time, we have developed a technology for visually establishing the optimum for each stage according to the picture of the differential kinetics of the intensity of luminescence in the direction "(rise of chemiluminescence from zero)--(maximum or plateau reached)--(decline to zero)". From the gallery of images corresponding to a number of expositions, paintings with maximum luminous intensities, that is, in optima, were selected. In principle, it is possible to monitor each protein spot or band and a set of spots/bands in the track with a

serum sample. Chemiluminescence was recorded using a "*Bromide Ethydium*" light filter. The protein was additionally controlled using a *Coomassi* light filter.

Registration of protein fluorescence on a blot (separated proteins by IEF and then colored proteins by SYPRO Protein Blot Stain).

Such registration made it possible to localize the distribution of protein aggregates (excitation of fluorescence at 254 or 365 nm, and emission at > 600 nm). Further, a re-probing replacement of fluorescence with chemiluminescence of blots was possible.

2.2. The use of technology to identify an early biomarker of lesion depth by SLE against the background of the development of APS (accompanied by thrombosis) – subisotype C4A5-pI-3.8-BLOTpH7 component of the patient's C4 complement

1. The original multi-stage proposed technology was applied to search for biomarkers of rheumatic autoimmune diseases - SLE variants. Using the example of the C4A isotype region detected during IEF, it can be seen that the subisotypes of the C4A isotype continue to be dose-dependent under complement inactivation conditions (when diluting sera samples with distilled water). This indicates in favor of the fact that in the blood of patients with rheumatic autoimmune diseases there are subisotypes of the C4 complement component in the blood in connection with the disease - irreversible, non-excreting, accumulating protein in the blood, diagnostically significant biomarker aggregates with covalently bound C4b reacting with antibodies to C4.
2. The biomarker C4A5-pI-3.8-BLOTpH7 of the depth of systemic damage to the body with combined SLE+APS syndrome is unique and has no analogues in the literature. Thus, a biomarker is proposed that characterizes the deterioration of the disease, and not the fact of the disease itself. The marker will help to differentiate complex (combined) cases of rheumatic diseases.
3. In the area of the C4A5-pI-3.8 subisotype on blot-pH7, microheterogenicity was observed in the serum of patients with SLE+APS in the form of two diffuse close bands (a more pronounced band as more acidic one) in the range of pI 3.75-3.85. It can be assumed that the possibility of image registration of the patient ratio of IgM and IgG rheumatoid factors is opened. A further increase in the resolution of the applied technology is to enhance the discreteness of the images of protein bands. An increase in the discreteness of the separated protein aggregates can be achieved: a) by procedures for applying sera to the gel plate (to make the start more longer and less wide in the inter-electrode separation

direction in the track, to provide further modernization of semi-dry blotting); b) by replacing chemiluminescent (less stable in time) procedures with fluorescent registration (more stable), for example, using commercial conjugates of antibodies with fluorochrome.

4. Technologies of differential optimum of live chemiluminescence imaging are promising in order to search for early and weak signals important for the diagnostics of multifactorial systemic diseases and further standardization of patient sera. The technology is promising for the successful search for early and relatively weak immune signals of biomarker significance for any rheumatic diseases involving C4/C3 systems, namely, fragments of C4b and C3b reacting with glycoconjugate targets. The technology can be extended to blocks of two-dimensional (2D) electrophoresis in a PAG plate. If there are known image maps of proteins in the model sera of healthy donors and patients, we can expect a simplification of the procedure for diagnosing the status and stage of the disease, an increase in the number of detectable protein biomarkers of pathology, as well as the use of antibodies at the level of antisera preparations ("crude" sera preparations) to the components (one or more) of the complement.

3. CONCLUSIONS

3.1. A technology is proposed to search for early image chemiluminescent signals involving the participation of C4 complement component (C4A and C4B subisotypes) and diagnosing status in the development of rheumatic/autoimmune diseases, as well as biomarking/standardizing sera of patients.

3.2. Biomarker C4A5-pI-3.8-BLOTpH7 of the depth of systemic damage to the body with combined SLE+APS syndrome is unique and has no analogues in the literature. The marker will help to better differentiate cases of rheumatic and autoimmune diseases.

3.3. It is possible to study the spectra of biological activities of the identified biomarker - as a membrane-bound and/or solubilized preparation(s).

Disclosure of conflict of interest

The authors declare no conflict of interest.

REFERENCES

1. Lakhtin MV. Variants of the isotyping of the human complement component C4. Dissertation of the Candidate of Biological Sciences (as a manuscript). Specialty 14.00.36: Allergology and immunology. Moscow, 2008; 156. (in Russian). https://elibrary.ru/item.asp?id=19190279
2. Kohleisen B, Kalies I, Kölble K, Kalden JR. Patient-specific heterogeneity of antinuclear antibodies as revealed by an isoelectric focusing immunoblot system. *Scandinavian Journal Immunology*, 1987; 26(1): 71-8. doi: 10.1111/ j.1365-3083.1987.tb02236.x.
3. Jara LJ, Capin NR, Lavalle C. Hyperviscosity syndrome as the initial manifestation of systemic lupus erythematosus. *Journal Rheumatology*, 1989; 16(2): 225-30.
4. Stich O, Kluge J, Speck J, Rauer S. Oligoclonal restriction of antiviral immunoreaction in oligoclonal band-negative MS patients. *Acta Neurology Scandinavia*, 2015; 131(6): 381-8. doi: 10.1111/ane.12350.
5. Lakhtin M. V., Lakhtin V. M. Immunochemical functional analysis of glycoantigens in connection with their aggregation and supramolecular assembly: prospects for assessing the healthy status of blood antigens. *Prenosology and a healthy lifestyle* (Saint Petersburg, Russia), 2020; 2(27): 5-8. (in Russian). https://elibrary.ru/contents.asp?titleid=63399
6. Kingsmore SF, Thompson JM, Crockard AD, Todd D, McKirgan J, Patterson C, Fay AC, McNeill TA Measurement of circulating immune complexes containing IgG, IgM, IgA and IgE by flow cytometry: correlation with disease activity in patients with systemic lupus erythematosus. *Journal Clinical Laboratory Immunology*, 1989; 30(1): 45-52.
7. Panush RS, Katz P, Longley S, Yonker RA. Detection and quantitation of circulating immune complexes in arterial blood of patients with rheumatic disease. *Clinical Immunology Immunopathology*, 1985; 36(2): 217-26. doi: 10.1016/0090-1229(85)90123-0.
8. Kozlov LV, Vratskikh EV, Lakhtin VM, Lapuk VA, Dyakov VL. Enzyme immunoassay for the determination of IgM rheumatoid factor in sera of patients with rheumatoid arthritis. *Clinical laboratory diagnostics (Moscow)*, 2002; 9: 46.
9. Ballanti E, Perricone C, Greco E, Ballanti B, di Muzio G, Chimenti MS, Perricone R. Complement and autoimmunity. *Immunology Research*, 2013; 56(2-3): 477-91. doi: 10.1007/s12026-013-8422-y.
10. Savelli SL, Roubey RAS, Kitzmiller KJ, Zhou D, Nagaraja HN, Mulvihill E, Barbar-Smiley F, Ardoin SP, Wu YL, Yu CY. Opposite Profiles of Complement in

Antiphospholipid Syndrome and Systemic Lupus Erythematosus Among Patients With Antiphospholipid Antibodies. *Frontiers in Immunology*, May 7, 2019; 10: 885. doi: 10.3389/fimmu.2019.00885.

11. Sandhu V, Quan M. SLE and Serum Complement: Causative, Concomitant or Coincidental? *Open Rheumatology Journal*, Sep. 30, 2017; 11: 113-22. doi: 10.2174/1874312901711010113.
12. Mulvihill E, Ardoin SP, Thompson SD, Zhou B, Yu GR, King E, Singer NG, Levy DM, Brunner HI, Wu YL, Nagaraja HN, Schanberg LE, Yu C-Y. Elevated serum complement levels and higher gene copy number of complement C4B are associated with hypertension and effective response to statin therapy in childhood-onset systemic lupus erythematosus. *Lupus Science Medicine,* Jul. 31, 2019; 6(1): e000333. doi: 10.1136/ lupus-2019-000333.
13. Pereira KMC, Faria AGA, Liphaus BL, Jesus AA, Silva CA, Carneiro-Sampaio M, Andrade LEC. Low C4, C4A and C4B gene copy numbers are stronger risk factors for juvenile-onset than for adult-onset systemic lupus erythematosus. *Rheumatology (Oxford),* 2016; 55(5): 869-73. doi: 10.1093/rheumatology/kev436.
14. Pereira KMC, Perazzio S, Faria AGA, Moreira ES, Santos VC, Grecco M, Pereira N, Andrade LEC. Impact of C4, C4A and C4B gene copy number variation in the susceptibility, phenotype and progression of systemic lupus erythematosus. *Advances in Rheumatology*, Aug. 6, 2019; 59(1): 36. doi: 10.1186/s42358-019-0076-6.
15. Mathsson L, Ahlin E, Sjöwall C, Skogh T, Rönnelid J. Cytokine induction by circulating immune complexes and signs of in-vivo complement activation in systemic lupus erythematosus are associated with the occurrence of anti-Sjögren's syndrome A antibodies. *Clinical Experimental Immunology*, 2007; 147(3): 513-20. doi: 10.1111/j.1365- 2249.2006.03313.x.
16. Murdaca G, Orsi A, Spanò F, Faccio V, Puppo F, Durando P, Icardi G, Ansaldi F. Vaccine-preventable infections in Systemic Lupus Erythematosus. *Human Vaccination Immunotherapy*, 2016; 12(3): 632-43. doi: 10.1080/21645515.2015.1107685.
17. Lakhtin MV, Kozlov LV, Lakhtin VM, D'Yakov VL. Deficiencies in C4A and C4B isotypes of human complement revealed by isoelectrofocusing and chemical reactivity of activated forms. *Russian Journal of Bioorganic Chemistry*, 2007; 33(4): 431-5.