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EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

Analysis of the Specificity of Auto-Reactive Antibodies to Individual Fragments of the Extracellular Domain of Desmoglein 3 in Patients with Pemphigus Vulgaris

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A method for the analysis of the epitope specificity of auto-reactive antibodies to desmoglein 3 (Dsg3) using competitive ELISA has been developed. It is based on a two-stage solid-phase ELISA with initial "depletion" of auto-reactive antibodies against the studied epitope and subsequent quantitative assessment of antibodies against full-length extracellular domain Dsg3. The proposed approach for assessing the specificity of the autoimmune response in patients with pemphigus vulgaris can provide in the future the possibility to personalize the therapy using plasmapheresis by preliminary selection of the antigenic composition of the extracorporeal immunosorbent.

Key Words: *pemphigus vulgaris; desmoglein 3; autoimmunity; autoantibodies; specificity; ELISA*

Pemphigus vulgaris (ICD-10 code L10.0) is the most common and severe disease from the group of bullous disorders characterized by the formation of intraepithelial flaccid blisters and erosions developing in the skin and mucous membranes of the oral cavity, esophagus, and other organs [11]. A key element in the pathogenesis of pemphigus is the formation of auto-reactive antibodies (IgG) to the structural proteins of desmosomes of the stratified squamous epithelium, primarily to desmoglein 3 (Dsg3) [3]. Normally, this glycoprotein with a molecular weight of 130 kDa, a member of the desmosomal cadherin superfamily, provides calcium-dependent homophilic connection between adjacent epithelial cells, participates in the processes of proliferation, differentiation, and morphogenesis [5,8]. Binding of auto-reactive antibodies to Dsg3 sterically blocks desmoglein transintegration [7] and triggers intracellular signaling involving mitogen-activated protein kinase p38 [14], which leads to degenerative changes of spinous layer of the epidermis with the formation of a suprabasal fissure (bladder). Therefore, the development of methods for the detection and quantitative characterization of autoantibodies to Dsg3 as a tool for the diagnosis of pemphigus vulgaris and its differentiation from other bullous dermatoses is a pressing problem. Creation of recombinant protein technologies contributed to the solution to this problem. In particular, eukaryotic expression systems such as baculovirus-insect cell expression systems [1] or systems in mammalian cell lines HEK 293, CHO,

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etc. provided the formation of the most correct conformation and profile of Dsg3 glycosylation with the corresponding preservation of immunoreactivity [6,12].

The obtained full-length recombinant Dsg3 is used as a diagnostic antigen in some commercially available ELISA test systems [6,10,12], expressing the activity of auto-reactive antibodies in relative units (RU) per 1 ml serum or plasma. Further study of the immunopathogenesis of pemphigus vulgaris showed different intensity of the autoimmune response to individual EC1-EC5 epitopes of the extracellular Dsg3 domain that varied depending on the disease severity and prognosis [9,13]. At the same time, it is believed that antibodies to the distal N-terminal EC1 and EC2 subdomains showing low activity in ELISA are more pathogenic, while more reactive antibodies to the proximal C-terminal EC4 and EC5 subdomains are less pathogenic [2]. The need for such an assessment is also determined by the prospect of developing personalized immunotherapy of pemphigus vulgaris based on the extracorporeal immunoadsorption [4]. However, there are currently no available methods for studying individual immunoreactivity profiles for individual EC1-EC5 fragments of the extracellular domain of Dsg3.

The aim of this study was the development of a new version of ELISA for evaluation of the epitope specificity of auto-reactive antibodies to Dsg3 in patients with pemphigus vulgaris.

MATERIALS AND METHODS

Serum samples from 20 patients with clinically and laboratory verified pemphigus vulgaris (ICD-10 code L10.0) admitted for inpatient treatment at the State Scientific Center for Dermatovenereology and Cosmetology in 2019-2020 were used as the source of auto-reactive antibodies.The total level of antibodies to Dsg3 in samples was characterized using Anti-Dsg3 ELISA IgG kits (Euroimmun). From these serum samples, IgG preparation with activity of 200 RU/ml was further obtained. The samples were stored at -70°C; before use, the samples were thawed at 4°C and diluted 1:100 with PBS (pH 7.4) containing 0.5 mM $CaCl_2$ (PBS-Ca), 0.05% Tween-20, and 1% casein.

Recombinant proteins reproducing the full-length extracellular domain of Dsg3 (Dsg3-full) or its separate EC1, EC2, and EC4 fragments (subdomains) were used as the antigens. The created expression constructs cloned at the NheI/XhoI sites into the pcDNA 3.4 vector provided synthesis of the mentioned proteins carrying human IgG1 Fc domain at the C-terminus for stabilization and the leader peptide (MMGLFPRTTG-ALAIFVVVILVHG) and Kozak sequence (CTCAAA) at the N-terminus. The target products were obtained by transient expression in Chinese hamster ovary cells (CHO line) and purified using affinity chromatography on protein A-sepharose. The obtained recombinant proteins in various concentrations were separately immobilized in the wells of high sorption plates (Greiner-bio) in PBS-Ca buffer. At the end of sorption, free binding sites in the wells were blocked with PBS-Ca solution with 0.05% Tween-20 and 5% casein (60 min, 37°C) and then washed with PBS-Ca.

Antigen-antibody interaction was carried out under controlled conditions in a thermostatic shaker (Thermo Scientific). The detection was carried out by adding a peroxidase conjugate to the χ-chains of IgG (Rosmedbio) excluding the possibility of binding to the Fc-domain in the composition of the recombinant proteins. Tetramethylbenzidine (Sigma-Aldrich) was used as the peroxidase substrate. Optical density was measured on a Multiskan FC microplate photometer (Thermo Scientific) at 450 nm (OD_{450}).

The results were processed statistically y ANOVA using Microsoft Excel. All experiments were performed in at least 5 replicates. The results are presented as $M \pm SEM$. The results obtained with the developed ELISA and commercial reference system were compared using the Student's t test at $p<0.05$.

RESULTS

First, we determined the optimal concentration of the antigen (full-length recombinant Dsg3) that allows quantitative detection of total autoantibodies to this protein with expression of activity in RU/ml similar to that in the reference test system Anti-Dsg3 ELISA IgG (Euroimmun). Analysis of the results of indirect ELISA in plates containing 0.1, 0.25, 0.5, 1.0, 1.5, and 2.5 μg sorbed Dsg3-full per well and subsequently filled with pooled IgG preparation with activity of 200 RU/ml, revealed a dose-dependent increase in OD_{450} with maximum values at a 10 μg/ml of Dsg3-full, followed by a "plateau effect", where further increase in the antigen concentration was no longer accompanied by a proportional increase in OD. In turn, comparison with the reference test system demonstrated high convergence of the results obtained with a comparable quantitative content of Dsg3 in the wells, but showed lower absolute OD_{450} values (0.4 *vs* 2). It simultaneously provided the possibility of carrying out the following quantitative measurements on a more extended linear section of the calibration curve.

The obtained result was decisive for solving the main problem: specification of the epitope specificity of auto-reactive antibodies in patients with pemphigus vulgaris. The solution was based on the principle of competitive ELISA: conducting the primary

interaction of the analyzed serum with recombinant proteins corresponding to individual EC1, EC2, and EC4 subdomains followed by assessment of activity of unreacted antibodies against full-length extracellular domain of Dsg3. However, an attempt to implement this principle in a variant involving interaction of the studied serum samples with individual recombinant proteins-subdomains in a concentration range of 7-55 μg/ml in the liquid phase (at 24°C for 3 h), their transfer and testing of Dsg3-full on the solid phase, led to a paradoxical result — an increase in the final OD_{450} values, which turned out to be 0.1-0.5 RU higher than those when testing intact serum samples. This phenomenon can be explained by the formation of homophilic contacts between the used subdomains in the mixture and the full-size protein on the solid phase, which led to an increase in the total presence of antigenic determinants in the ELISA plate and was an insurmountable limitation for using the proposed approach.

An alternative solution was competitive depletion of autoantibodies to the EC-construct adsorbed on the solid phase, which ruled out the possibility of further homophilic EC:Dsg3 interaction. For this, a set of plates was prepared: wells of plate 1 contained separately sorbed Dsg3-full, EC1, EC2, and EC4 as well as BSA to control nonspecific sorption; plate 2 contained sorbed Dsg3-full as described above (Fig. 1, *a*).

The principle of their use when performing a two-step competitive ELISA is initial depletion of antibodies of the studied serum at a specific epitope in plate 1 and subsequent transfer of unreacted antibodies to plate 2, where their residual amount is estimated relative to the intact serum of the same patient (Fig. 1, *b*). The results of ELISA in plate 1 allows determining direct reactivity of the serum to certain epitopes; however, in the absence of calibrated mono- or polyclonal autoantibodies of similar specificity, it cannot be quantitatively characterized and expressed in OD_{450} values (RU). In turn, OD_{450} values in plate 2 can be directly converted into activity values (RU/ml) and compared with the same value previously determined in the same test system for intact serum. In this case, the options for the expected result are "high OD_{450} values in plate 1/low OD_{450}values in plate 2" (high activity against the analyzed epitope) or "low OD_{450}values in plate 1/ high OD_{450} values in plate 2" (low activity against the analyzed epitope).

The developed method with the optimal concentrations of reagents and incubation conditions determined in individual experiments can be presented as follows: 1) the analyzed serum samples are diluted 1:100 with PBS-Ca containing 0.05% Tween-20 and 1% casein, after which 100-μl aliquots are added to wells of plate 1 with individually adsorbed Dsg3-full, EC1, EC2, EC4, and BSA and incubated 3 h at 25°C under stirring at 200 rpm; 2) after incubation, the analyzed samples are transferred from wells of plate 1 to wells of plate 2 with sorbed Dsg3-full. Special wells in plate 2 contain calibration samples (serum samples from patients with pemphigus vulgaris) with activities of 20 and 200 RU/ml measured using the reference test system. Plate 2 is incubated as described above (1 h, 25°C, 200 rpm); 3) after incubation, the serum is removed, plate 2 is washed three times with PBS-Ca containing 0.05% Tween-20 and 0.3 M NaCl. After that, 100-μl aliquots of peroxidase conjugate to IgG χ-chains are added to each well and incubated at 25°C and 200 rpm for 1 h; optionally, similar actions are performed with plate 1; 4) the plates are washed again three times with PBS-Ca containing 0.05% Tween-20 and 0.3 M NaCl; 100 μl tetramethylbenzidine hydrochloride substrate solution is added to the wells for 30 min (25°C, 200 rpm). The reaction is stopped by adding 100 μl of 4 N sulfuric acid solution; 5) OD_{450} is measured and calibration curve is constructed

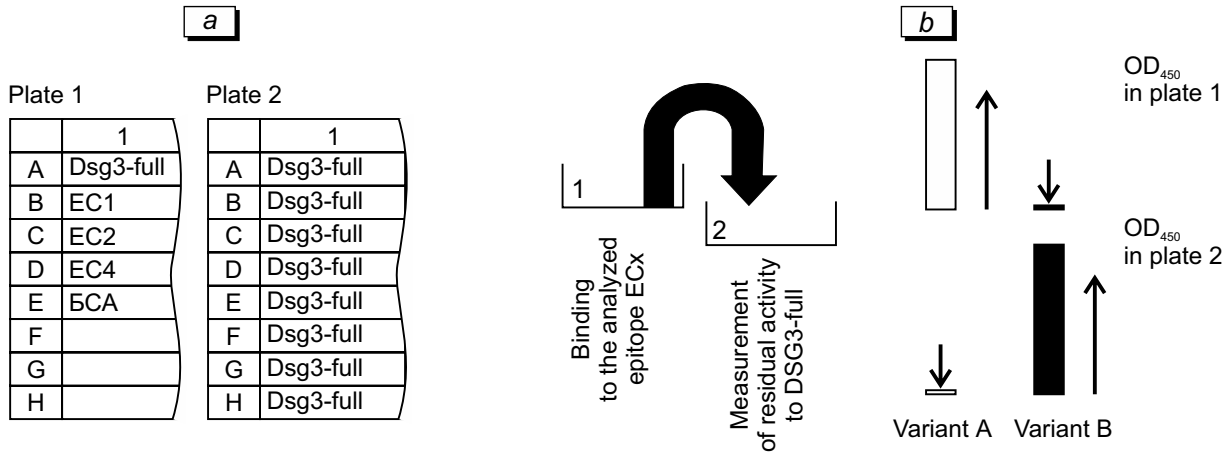

Fig. 1. A complete set of ELISA plates (*a*) and an algorithm for their use (*b*) when assessing the profile of the epitope specificity of the serum of patients with pemphigus vulgaris and assessing the reactivity of serum to certain epitopes.

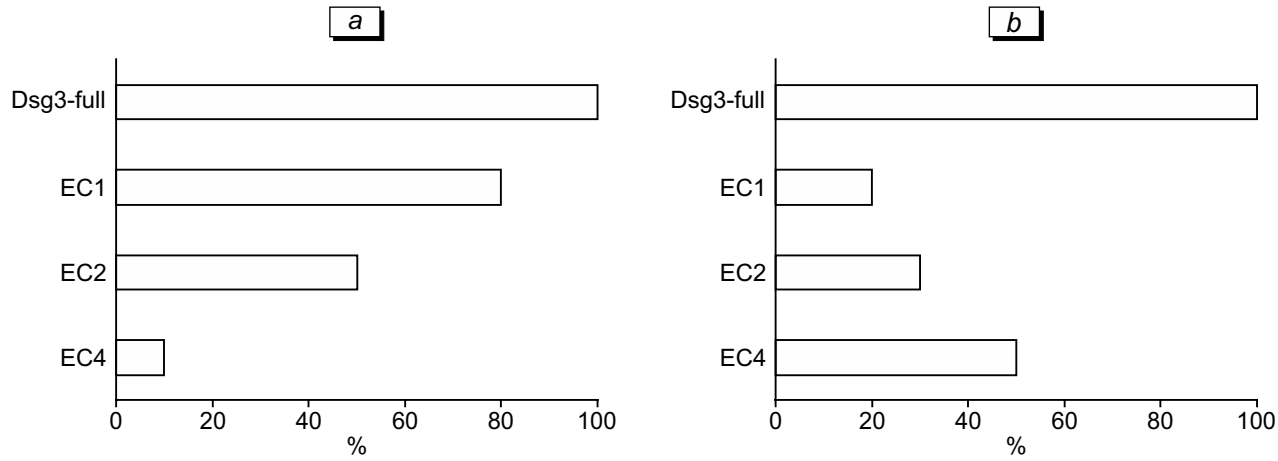

Fig. 2. Profiles of immunoreactivity to the studied subdomains in serum samples from patients with pemphigus vulgaris.

based on OD of calibration samples with activities of 20 and 200 RU/ml, which allows to quantitatively characterize activity of each analyzed serum. Finally, based on a comparison of the RU/ml values of each individual serum previously incubated in plate 1 and subsequently analyzed in plate 2, the degree of its specific binding (competition coefficient) with respect to individual epitopes of desmoglein3 is calculated using the formula:

$$[1-(APos-AR)/(APos-ANeg)]\times 100\%,$$

where AR is the activity of serum after preincubation in the well of plate 1, immobilized with a recombinant protein — a EC1, EC2, and EC4 subdomain; APos is the serum activity after preincubation in the well of plate 1 with the recombinant full-length extracellular domain Dsg3-full; ANeg is the serum activity after preincubation in the control well of plate 1 with immobilized BSA.

The correctness of the obtained result is assessed by its comparison with the OD values in the plate 1, which qualitatively evaluates the analyzed activity.

Testing of the developed two-stage competitive ELISA was carried out in a group of 20 patients with clinically and laboratory verified diagnosis of pemphigus vulgaris. Evaluation of the immunoreactivity of serum samples obtained made it possible to show a diagnostically significant presence of autoantibodies to Dsg3-full (>20 RU/ml) in 19 patients; their absence in 1 patient could be explained by the development of a humoral autoimmune response to other components of desmosomes. At the same time, the mean activity was 346.5±84.3 RU/ml (range from 10 to 1500 RU/ml). Analysis of the epitope specificity spectrum of autoantibodies showed a significant activity variation in relation to the individual subdomains (Fig. 2). Pronounced autoimmune response to distal EC1 and EC2 subdomains was most common (in 11 and 1 patients, respectively), more than 50% autoantibodies had this epitope specificity (Fig. 2, *a*). In one patient, a predominant response to one of the proximal domains, EC4, was found (Fig. 2, *b*). Finally, auto-reactive antibodies in 6 patients equally interacted with all analyzed epitopes. It should be noted that the sum of reactivity to individual Dsg3 subdomains typically exceeded 100%, which suggests the existence of the phenomenon of their cross-reactivity with individual analyzed epitopes and requires further study.

This study for the first time demonstrates the possibility of analysis of the epitope specificity of autoantibodies to Dsg3 using the competitive ELISA and shows the existence of differences in the spectra of this specificity in patients with pemphigus vulgaris. The originality of the proposed approach is the two-stage implementation of solid-phase ELISA, which provides the initial "depletion" of auto-reactive antibodies against the studied epitope and the subsequent quantitative assessment of unreacted antibodies against the full-length extracellular domain Dsg3. Further development of the proposed approach associated with the clarification of the specificity of the autoimmune response in patients with pemphigus vulgaris, which should provide in the future the possibility topersonalize the therapy using plasmapheresis by preliminary selection of the antigenic composition of the extracorporeal immunosorbent.

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